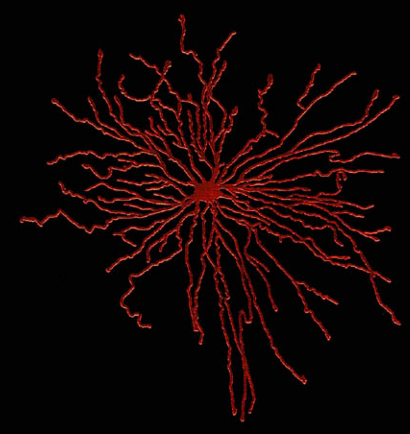
NEUROGLIA



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To those who believed in glial cells
during the long, dark period
when the neuron concept dominated brain science.

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Preface

Non-neuronal cells, termed neuroglia, were recognized as independent elements of the nervous system nearly a century and a half ago by Virchow. These cells are present in primitive nervous systems and, undoubtedly driven by positive evolutionary pressures, have persisted in high density and acquired greater diversity in mammals. Knowledge about glial cells has accumulated at a phenomenal rate in the last 30 years, and has become relevant to all fields of neurobiology. With so much new information at hand, we felt that this was an important time to assemble the facts about these cells as we presently understand them.

Historically, glial cells were viewed as a type of CNS connective tissue whose main function was to provide support to the true functional cells of the brain, the neurons. This firmly entrenched concept remained virtually unquestioned for the better part of a century. But glial cells are neither connective tissue nor mere supportive cells. In contrast to early beliefs, glial cells are now recognized as intimate partners with neurons in virtually every function of the brain, and as participants in the pathophysiology of the dysfunctional or diseased brain. These cells have been challenging to study, however, because their functions are not associated with easily recorded electrical signals as is the case with neurons.

While books about the nervous system have grown in size and complexity attempting to accommodate the frantic production of new neuroscience information, the incorporation of new facts about glia has not kept pace. One simply can not learn about glial cells by turning to the typical neuroscience textbook (*From Neuron to Brain* by Nicholls, Martin and Wallace, is a notable exception). This curious fact has also been a motivation for bringing together in the present volume a detailed summary of what is currently known about these cells. It will, we hope, also encourage better integration of the glial and neuronal information bases, which each suffer in the absence of the other. The brain can not be understood as the functional sum of two isolated cellular compartments; it must, we think, be seen as a single entity containing neurons and glial cells working in seamless harmony with one another. Somehow, this essential message has gone too long undelivered.

Glial research is at a particularly exciting point in its evolution. Great advances in our knowledge about nervous system diseases have opened the door for thinking about the role of glial cells in the pathogenesis of these conditions and in their treatment. Therapies that would literally have been the stuff of science fiction only a decade ago are now in advanced stages of testing. Patients with Parkinson's disease who no longer respond to our best medicines, for example, have received brain tissue transplants, whose effectiveness may be enhanced by including glial cells as factories for the production of trophic substances. Glial transplants to refurbish areas of demyelination may also be possible in the near future. Our capacity to measure the brain's functional molecules and determine their cellular topography has revealed a baffling array of neurotransmitters, receptors, ion channels, adhesion molecules, and trophic factors associated with glial cells. These findings are stimulating and broadening the field of glial research. They provide critical insights about how neurons and glial cells might communicate with each other, and reveal an astonishing overlap between the features of the brain's two principle cell types that would have been heresy not long ago.

The many experts who wrote chapters for this volume contributed in other val-

uable ways as well. Before the writing began, they provided invaluable advice about what topics should be covered. In an unselfish manner, they adjusted the scope of their individual contributions so that they fit the context of the book as a whole. To enhance the quality and utility of the chapters, each underwent a stage of peer review and this was cheerfully provided by other authors. The editorial burden was significantly lightened by the satisfaction of dealing with this uniquely talented and energetically committed group of authors. They shared our view of the importance of developing a compendium volume about glial cells, and continuously reinforced our enthusiasm for the project as it moved forward. We acknowledge their essential partnership in the making of Neuroglia, and thank them for their efforts.

One point should be made in concluding. As impressive as our gains in glial cell knowledge have been, the best is yet to come. Glial researchers have struggled with our own version of the Heisenberg uncertainty principle: How to study the role of glial cells in the multicellular actions of the nervous system without interfering with the very functions we wish to understand? Our initial efforts were a compromise. We retreated somewhat from the immense complexity of the intact nervous system in favor of simplified preparations that allowed more rigorous study. This reductionistic approach has produced a mountain of provocative information, as detailed here, but few definitive answers. Consequently, we have long lists of glial cell properties while the list of proven functions is small. But starting with these demonstrated properties, testable hypotheses of glial cell function can now be formulated with greatly improved precision, taking full advantage of new or refined research technologies. A rich yield of vital new insights about the functions of neuroglia should follow and future editions of this book will survey those benefits.

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Morphology

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1 Morphology of astrocytes

ALAIN PRIVAT, MINERVA GIMENEZ-RIBOTTA, AND JEAN-LUC RIDET

It is generally acknowledged that Virchow (1846) discovered the existence of another element, besides neurons, in the central nervous system. However, the first account of neuroglia was probably that of Dutrochet (1824), who described small globules (*corpuscules globuleux*), he observed in great quantities on the surface of large globules (*cellules globuleuses*), in the nervous system of mollusks. But Virchow used the term *Nervenkitt*, which was later translated as *neuroglia*, to account for "a connective substance formed in the brain and the spinal cord in which the nervous system elements are embedded." Even though our present concepts have been modified to a large extent, Virchow's original description of a tissue that is able to fill all spaces between nerve elements still holds true. The major contribution of Deiters (1865), was to view those cells that do not have an axon as nonneuronal cells. Two of his original pictures, reproduced by Somjen (1988), could correspond either to small neurons or to oligodendroglia. However, credit for the modern study of glial cells should probably be given to Golgi (1885), whose technique permitted for the first time a reliable *morphological* identification of all cell types in the nervous system of vertebrates.

The distinction between fibrous and protoplasmic astrocytes, often attributed to Ramón y Cajal (1909), was in fact made by Andriezen (1893), who distinguished fibrous glia, found essentially in the white matter, from protoplasmic glia found in the gray matter. This distinction between gray and white matter glia was later substantiated by Kölliker (1893), Azoulay (1894), and Retzius (1894). Ramón y Cajal, first using Weigert stain (1895), then his own gold chloride sublimate method (Ramón y Cajal, 1913) adopted this classification, and he attached the name of "astrocyte" to both cell types. The Weigert stain was especially useful at that time as it permitted Ramón y Cajal to substantiate the difference between fibrous and protoplasmic astrocytes: gliofibrils appeared to be absent in the protoplasmic astrocyte. Later, the gold chloride sublimate method enabled him to recognize that fibrils were present in astrocytes of both gray and white matter.

The extensive use of Golgi's technique enabled Ramón y Cajal to describe a "third element," different from neurons and astrocytes, which was later found by Rio Hortega (1919, 1920), using the capricious silver carbonate methods, to be composed of two different cell types: microglia and oligodendrocytes. All these metallic impregnation techniques led Rio Hortega (1921, 1922, 1933), and Ramón y Cajal (1920), to describe a wide variety of shapes and sizes including a type of "intermediate astrocyte," which had been found in the cerebellum by Terrazas (1897). At the same time, Penfield (1924, 1932) attempted to correlate the morphological characteristics of glial cells, as revealed by metallic impregnations, with the nuclear and cytoplasmic characteristics of those same cells observed on paraffin sections stained with classical histological dyes.

Regarding the possible function of astrocytes, Golgi (1885), noticed the frequent contact of glial processes with blood vessels on the one hand, and with nerve cells on the other, and attributed to them the function of supplying nutrients to the cell body and the axon of nerve cells. His (1889) made the suggestion that embryonic glial cells guide the migration of developing neurons.

In a remarkable essay, Lugaro (1907) adopted the suggestion of His, and made the proposal that adult astrocytes keep the interstitial milieu compatible for the function of neurons. He added that these cells could serve to "chemically split or take up" substances released by nerve cells to ensure communication and excite one another, thus "buffering" their communication in time and space. Ramón y Cajal (1909), rejected Golgi's nutritive theory, and, although he admitted his own ignorance, due essentially to the lack of proper tools to study glial cells directly, he hypothesized that glial cells and their processes serve to insulate nerve fibers, and eventually they proliferate to fill the gaps left by dying neurons in pathological conditions.

In the second half of the twentieth century, the advent of electron microscopy enabled several investigators (Luse, 1956; Farquhar and Hartmann, 1957; Bunge et al., 1960; De Robertis and Gershen-

feld, 1961; Palay et al., 1962), to analyze the ultrastructure of astrocytes, and to describe, among other organelles, the ultrastructural appearance of gliofibrils in fibrous astrocytes. Herndon (1964), and Mugnaini and Walberg (1964), observed that protoplasmic astrocytes also contained gliofilaments. Mori and Leblond (1969) adapted the gold chloride sublimate technique of Ramón y Cajal to electron microscopy, and they found that the metallic deposit was concentrated over gliofilaments.

The biochemical nature of gliofilaments was disclosed by Eng et al. (1971), and Bignami et al. (1972), through the identification of glial fibrillary acidic protein (GFAP). It was then shown by Eng and Kosek (1974), Schachner et al. (1977), and Eng and Bigbee (1978), to be associated to the 8 to 10 nm intermediate filaments of astrocytes, together with vimentin (Dahl et al., 1981; Schnitzer et al., 1981). Nevertheless, numerous astrocytes in the normal brain are negative for GFAP immunocytochemistry (Ling and Leblond, 1973), and GFAP-negative astrocytes predominate in the gray matter (Bignami and Dahl, 1976; Ludwin et al., 1976; Kitamura et al., 1987).

Other specific cell markers detected by immunocytochemistry include the S-100 protein, identified by Moore (1965), which is preferentially expressed in astrocytes (Matus and Mughal, 1975; Ludwin et al., 1976; Cocchia, 1981; Ghandour et al., 1981), the C1 antigen, which characterizes mainly Bergmann glia (Golgi epithelial cells), and retinal Müller cells in adult mammals (Sommer et al., 1981), and the M1 antigen (Lagenaur et al., 1980), which is specific for protoplasmic and fibrous astrocytes.

A special mention should be made of the A2B5 and Ran-2 antibodies, extensively used by Raff and colleagues (Bartlett et al., 1981; Raff et al., 1983a, 1983b, 1984a, 1984b; Miller and Raff, 1984). The monoclonal antibody A2B5, which recognizes many gangliosides in nerve tissue (Fredman et al., 1984), was used initially *in vitro* to differentiate two classes of astrocytes (Raff et al., 1983a). Type 1, present in cultures developed from prenatal optic nerve, is devoid of A2B5 immunoreactivity; type 2, found in cultures derived from postnatal optic nerve, shares the positivity for A2B5 with oligodendrocytes and a common progenitor. This led Miller and Raff (1984) to claim that A2B5 could distinguish inbetween fibrous and protoplasmic astrocytes. However, type 2 astrocytes have never been found *in situ* in the nervous system of vertebrates (Privat and Rataboul, 1986; Skoff and Knapp, 1991). The latter authors, through a very careful combined autoradiographic and immunocytochemical study, have demonstrated that the wave of prenatal generation of astrocytes, claimed by Miller et al. (1985) and based on *in vitro* results, did not exist *in vivo*. Recent studies by Noble (1991) and Espinosa de los Monteros and Zhang (1993), confirmed the view of Mori and Leblond (1969), Privat and Leblond (1972), Paterson et al. (1973), and Privat and Fulcrand (1977), that astrocytes and oligodendrocytes arise from separate precursors, which are the progeny of a common ancestor, located in the ventricular layer before birth and in the subependymal layer postnatally (Smart, 1961; Privat and Leblond, 1972; Privat, 1975; Levison and Goldman, 1993).

Thus, this controversy underlies the enormous gap that exists between *in vivo* studies, which combine ultrastructural examination of cell characteristics with detection of specific epitopes and analyze the actual characteristics and fate of given cell type, and *in vitro* studies, which are best able to explore what cells can do when transported in an artificial environment (Privat et al., 1979). In addition, it is noteworthy that once astrocytes are plated in a culture dish, they can be considered initially as reactive (Bignami and Dahl, 1989). Whatever their morphology (i.e., fibrous or protoplasmic), cultured astrocytes are strongly GFAP-immunoreactive (Figure 1-1).

The goal of this chapter is to describe the morphological characteristics of astrocytes *in vivo*, stressing their diversity of shapes and sizes, and comparing classical metallic impregnations, electron microscopy, and immunocytochemistry.

We have chosen to organize our description around one region of the central nervous system—the spinal cord—where one can demonstrate, in the limited surface of a cross section, radial glia, fibrous and protoplasmic astrocytes, and even subclasses of the latter. However, the different types of astrocytes will also be illustrated in various regions of the brain, such as cerebral cortex, cerebellum, corpus callosum, whenever necessary for the purpose of our description.

MORPHOLOGY OF ASTROCYTES

Light Microscopy

Examination of sections impregnated with the technique of Golgi, modified by Rio Hortega (1918), permits the distinction of three main types of astrocytes according to their spatial organization:, (1) *radial cells*, which are disposed in a plane perpendicular to the axis of the ventricles, and span the whole thickness of the white matter (Figure 1-2A), (2) *fibrous, nonradial astrocytes*, located also in the white matter, which send their processes in various direc-

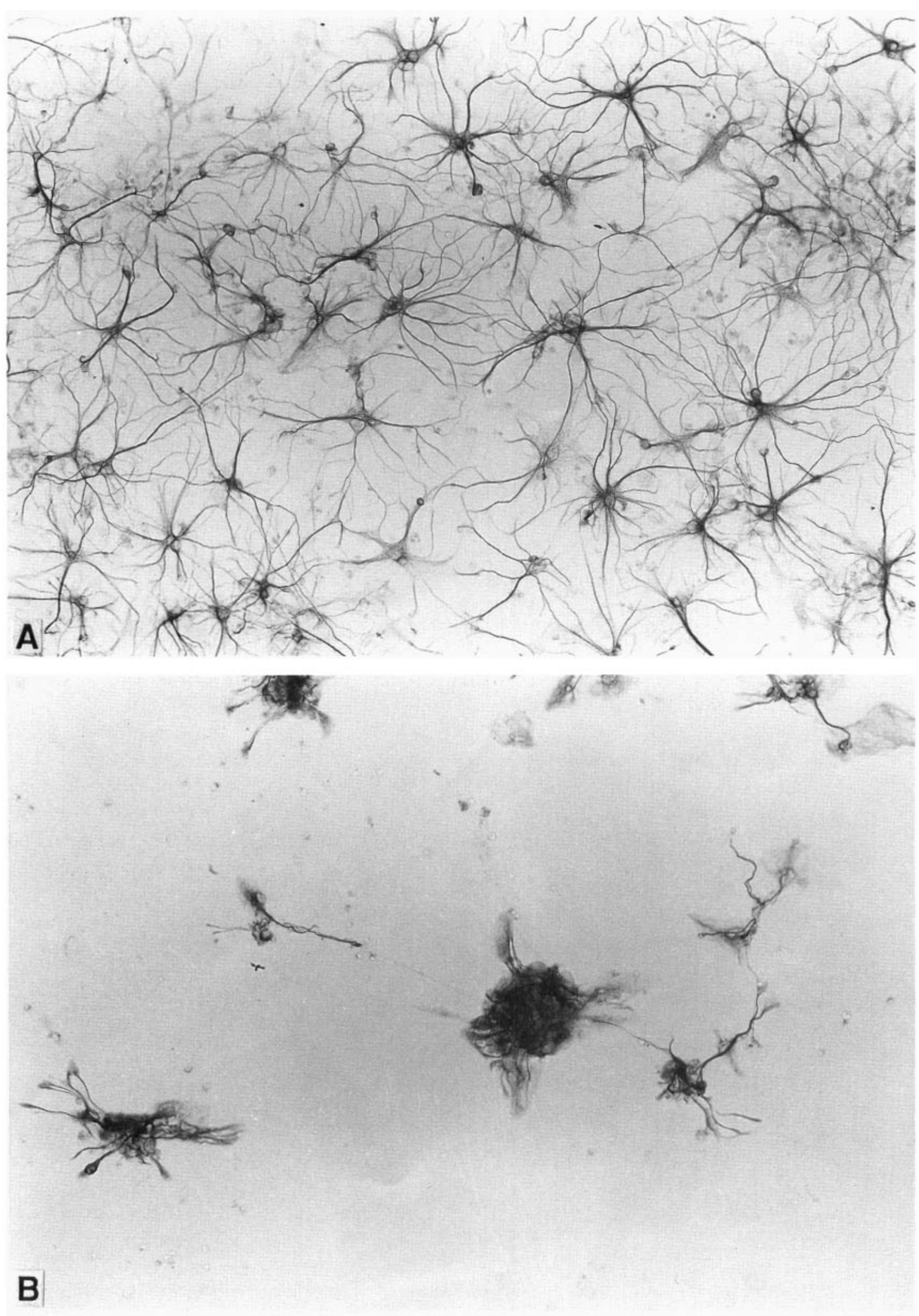


FIG. 1-1. (A) Primary culture of astrocytes from cerebral cortex of day 18 rat fetuses, light microscopy, GFAP immunodetection. Numerous fibrous astrocytes are intensely immunostained. ×250. (B) Primary culture of astrocytes from cerebral cortex of day 18 rat fetuses, light microscopy, GFAP immunodetection. Immunoreactive protoplasmic astrocytes. ×250.

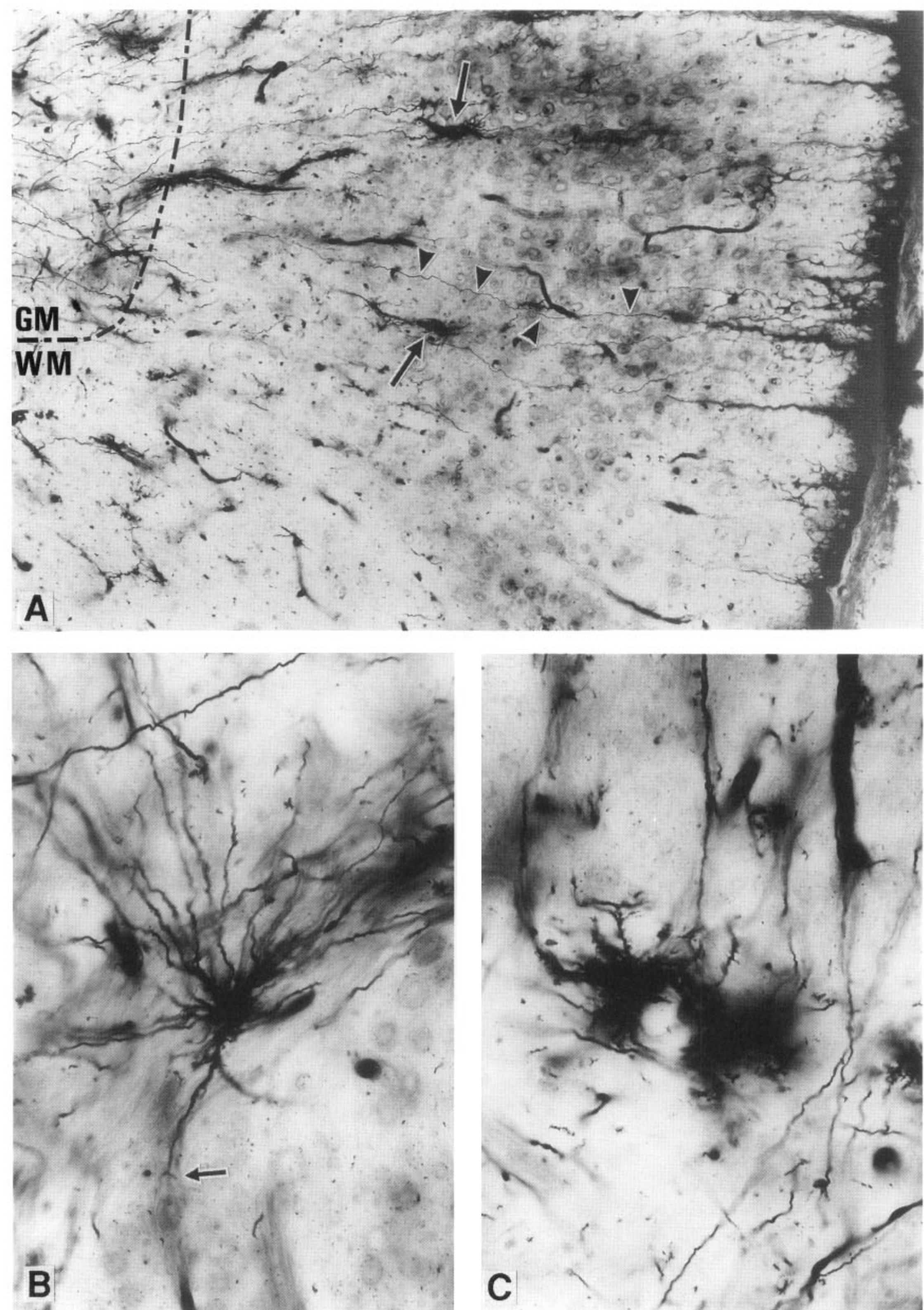


FIG. 1-2. (A) Cat spinal cord white matter, light microscopy, Golgi-Rio Hortega impregnation. Thin long unbranched radial processes (*arrowheads*) can be seen coursing throughout the whole white matter from the pia mater to the gray matter. Two radial astrocytes are clearly observed (*arrows*). *GM*, gray matter; *WM*, white matter. ×125. (B) Cat spinal cord white matter, light microscopy. Golgi-Rio Hortega impregnation. Stellate fibrous astrocyte with long unbranched processes. *Arrow* points to a vascular endfoot. ×375. (C) Cat spinal cord gray matter/white matter border, light microscopy, Golgi-Rio Hortega impregnation. Two protoplasmic astrocytes, characterized by a spherical perikaryon and numerous short and spiny processes, extend a few long fibrous processes (at the top of the figure), in the white matter. ×375.

tions and are not in contact with the pia mater (Figures 1-2B and 1-4B), and (3) *protoplasmic astrocytes*, located in the gray matter, which are characterized by short, ramified crimped processes (Figures 1-2C, 1-3B, and 1-4A). Although transitional forms between the first two types are not rare, we have found only rare evidence of intermediate forms between protoplasmic astrocytes and fibrous ones, which are usually located at the interface of the gray and white matter.

Radial Astrocytes. As described by Ramón y Cajal (1909), with the standard Golgi technique and the method of Weigert, radial astrocytes impregnated with the technique of Golgi-Rio Hortega span the entire white matter, with one process or a group of processes abutting onto the pia matter, and other processes deeply buried in the gray matter. The perikaryon of these cells is located either in close vicinity of the pia matter or at some distance from it. The processes are unbranched, rectilinear, often running in bundles into the fibrous axes of the white matter (Figure 1-2A).

After immunodetection of GFAP (Figure 1-5A), radial astrocytes are readily detected as fibrous axes coursing through the white matter of the lateral and ventral fasciculi. Upon close examination, they do not appear as smooth and unbranched as with the Golgi-Rio Hortega impregnation. Rather, they appear prickly, and somehow irregular in caliber. The perikaryon is usually only partially stained, possibly corresponding to the perinuclear organization of gliofibrils.

After immunodetection of S-100 protein (Figure 1-6A), which is a cytosolic soluble calcium-binding protein (Baudier et al., 1982, Donato, 1986), the perikarya of radial astrocytes and the proximal part of their processes is intensely impregnated. The surface of the cells and processes appears also prickly, as with GFAP. At variance with the latter, if thin processes are stained, they can rarely be followed in continuity with longer processes.

On semithin sections stained with toluidine blue (Figure 1-7), bundles of myelinated axons appear separated by radial, lightly stained axes, along which one can identify light, elongated, sometimes crenated nuclei, whose nuclear envelope is outlined by a thin rim of chromatin. Immunodetection of GFAP evidences a distinct reaction around those nuclei, extending in to the radial axes. These cytological characteristics are in accordance with those described by Ling and coworkers (1973), in the rat corpus callosum, by Sasaki and coworkers (1989), in the cat spinal cord, and are currently used for the identification of white matter astrocytes in semithin sections (Skoff and Knapp, 1991). With this technique, the boundaries of the cytoplasm are not precisely detected; the radial axes in which these astrocyte nuclei are embedded are lightly stained with toluidine blue, contrasting with neighboring oligodendrocytes whose cytoplasm appears denser (Figure 1-7A).

Radial glial cells are present in other locations in the central nervous system of vertebrates. Three cell types have been described in adult animals.

Müller Cells of the Retina. Müller cells are elongated throughout the whole thickness of the retina, from the inner to the outer limiting membrane. Their outer segments are generally stout, extensively anchored together to form the external limiting membrane. Their nucleus is located in the nuclear layer, and many processes radiate from their main axis in plexiform and nuclear layers. Finally, thin processes are inserted between the fibers of the nerve fiber layer, where they intermingle with those of a few astrocytes. In semithin sections, their nucleus appears elongated, sometimes notched with a homogeneous nucleoplasm, and a thin rim of chromatin along the nuclear envelope.

Golgi Epithelial Cells of the Cerebellum (Bergmann Glia). Golgi epithelial cells extend radially through the molecular layer of the cerebellar cortex. Their perikarya are located between those of Purkinje neurons, and they send an apical tuft of rectilinear processes, which end in contact with the pia mater. With metallic impregnations, these processes appear decorated with thin, irregular appendages (Figure 1-8A). At the surface of the cerebellar folia, the endfeet of Golgi epithelial cells constitute a continuous layer of glia limitans. On semithin sections stained with toluidine blue, the nuclei of Golgi epithelial cells appear spherical or ovoid, sometimes elongated along the axis of the folium, and are closely packed between those of Purkinje neurons.

Tanycytes. Tanycytes are radial glial cells whose nucleus is located in the ependymal lining of the ventricles or immediately beneath it. They are present all along the third ventricle, the aqueduct, the fourth ventricle, and the central canal of the spinal cord (for review see Rafols, 1986). With metallic impregnations, their basal process appears to terminated with an endfoot on the pia mater (Figure 1-3A). This is a unique, unbranched process of large caliber whose surface is irregular and which emits short expansions contacting nearby blood vessels (see Chapter 4 for details).

Fibrous Astrocytes. Fibrous astrocytes scattered in the white matter of the spinal cord are less numerous than radial ones. They appear with the metallic impregnation of Golgi-Rio Hortega as stellate cells

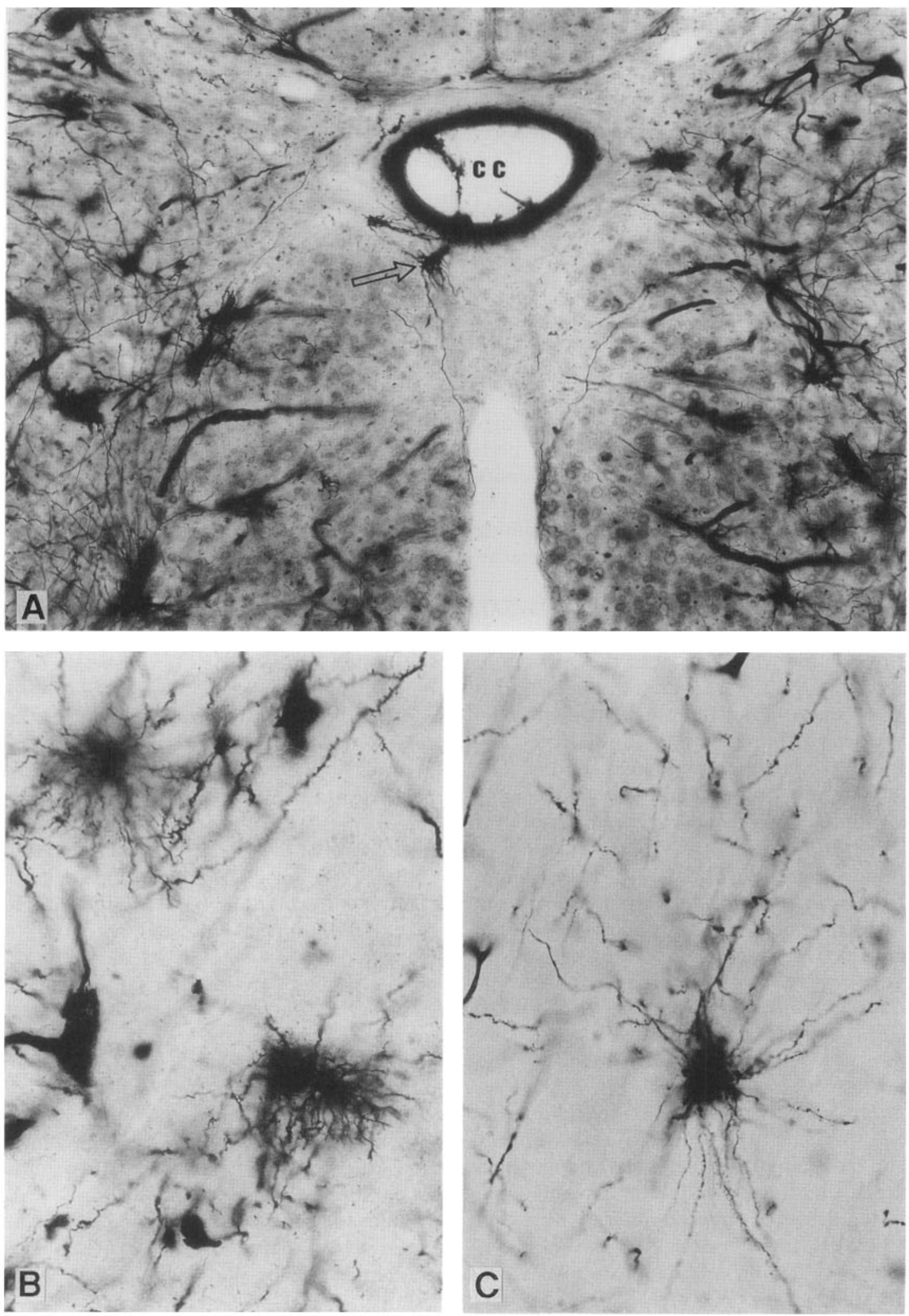


FIG. 1-3. **(A)** Cat spinal cord, light microscopy, Golgi-Rio Hortega impregnation. General view of the periependymal area; a tanycytelike cell (*arrow*), whose perikaryon is located in the vicinity of the central canal (*cc*), sends a process in contact with the pia mater. ×125. **(B)** Cat cerebral cortex gray matter, light microscopy, Golgi-Rio Hortega impregnation. Two protoplasmic astrocytes exhibit numerous short spiny processes. ×470. **(C)** Rat cerebral cortex gray matter, light microscopy, Golgi-Cox impregnation. A protoplasmic astrocyte shows some long thin, tortuous, and crimped processes. ×760.

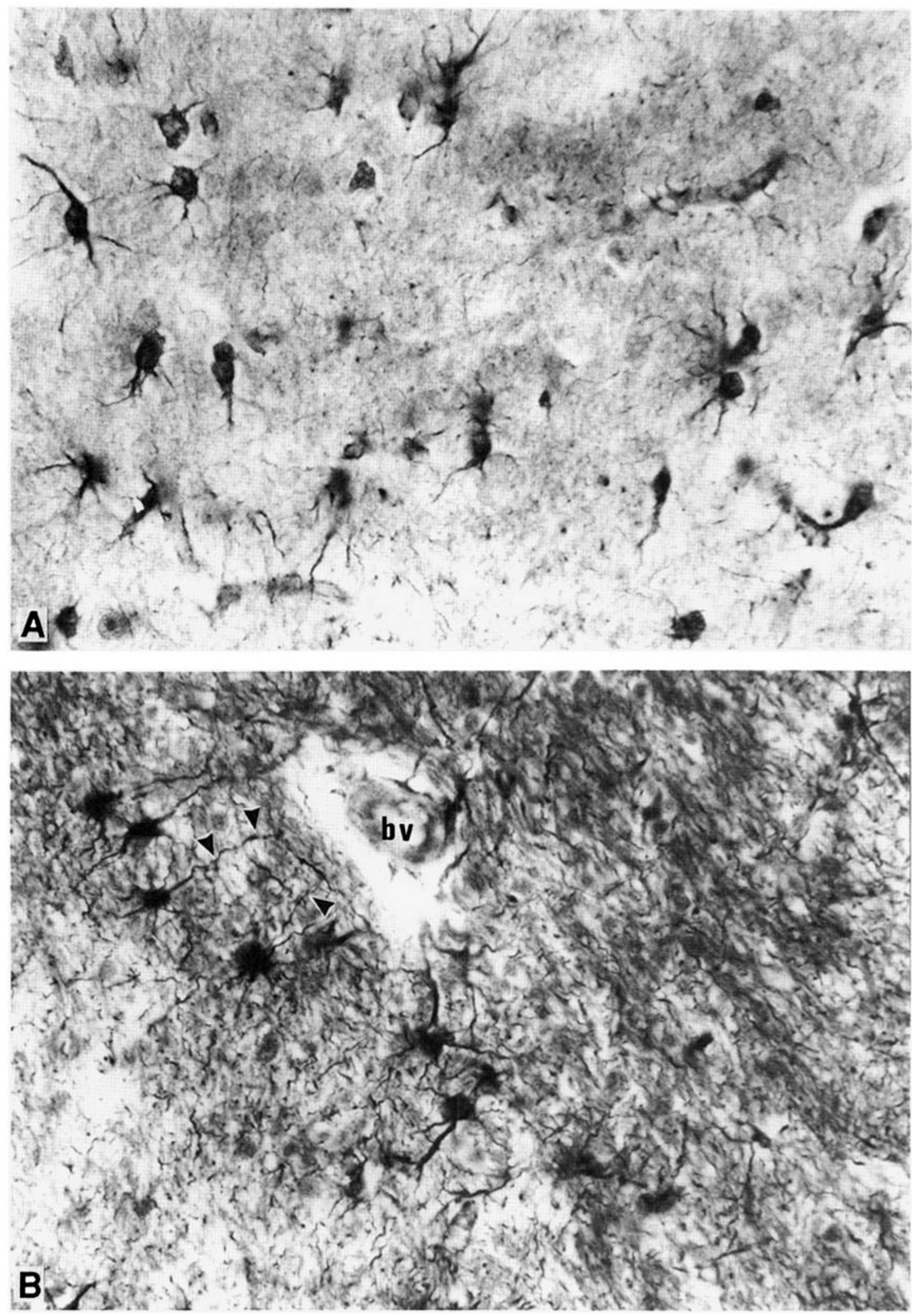


FIG. 1-4. **(A)** Cat cerebral cortex gray matter, light microscopy, gold chloride sublimate. Numerous protoplasmic astrocytes of which only a few processes are impregnated. ×360. **(B)** Cat cerebral cortex white matter, light microscopy, gold chloride sublimate. Numerous fibrous astrocytes with long unramified (*arrowheads*), processes. Apposed onto a blood vessel (*bv*), is a perivascular astrocyte. ×360.

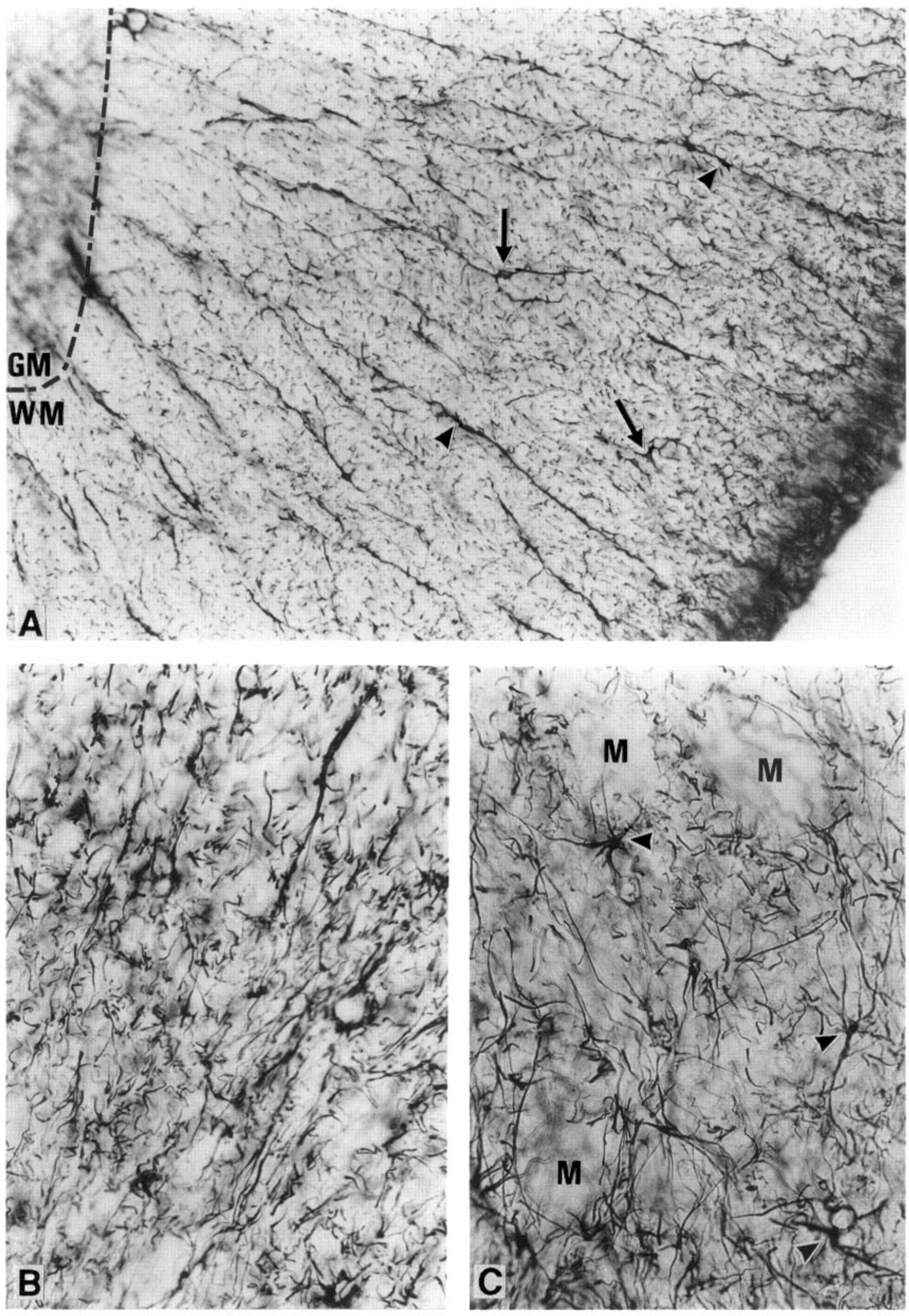
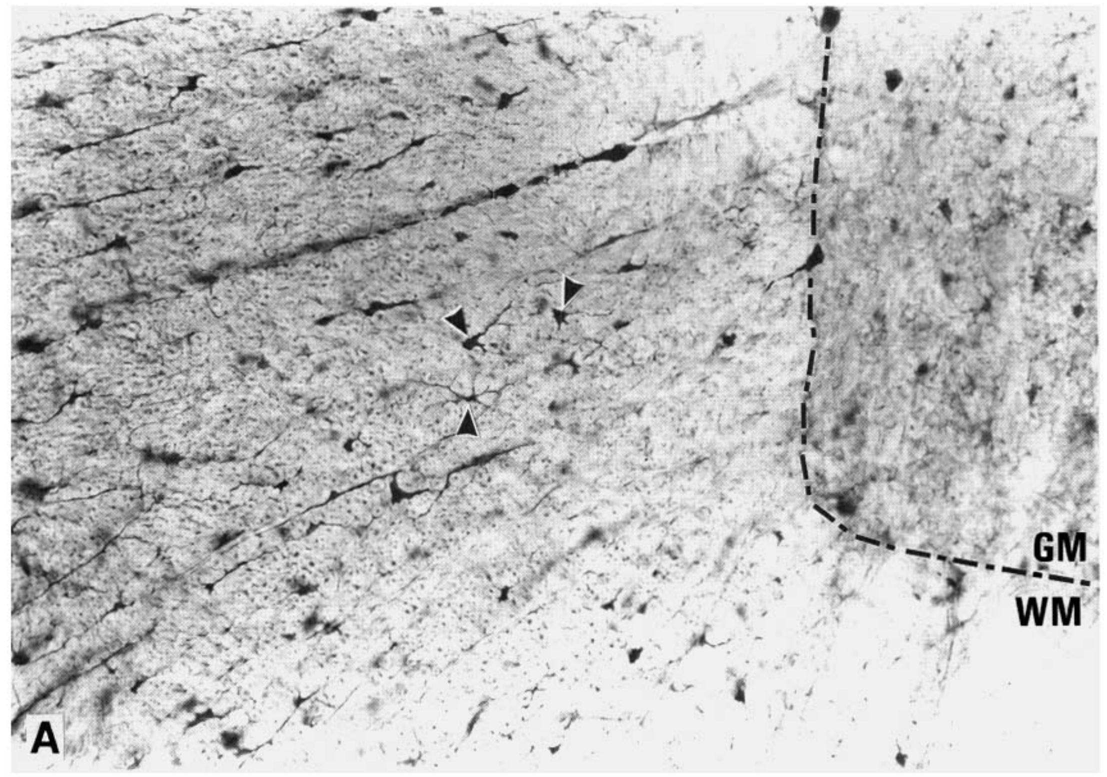


FIG. 1-5. (A) Rat spinal cord white matter, light microscopy. GFAP immunodetection. Both fibrous astrocytes (*arrows*), and radial glia (*arrowheads*), are immunostained. *GM*, gray matter; *WM*, white matter. ×250. (B) Rat spinal cord gray matter, light microscopy, GFAP immunodetection. High magnification of the dorsal horn showing numerous immunoreactive astroglial processes, whose main orientation is anteroposterior in a horizontal plane; few perikarya are observed. ×530. (C) Rat spinal cord gray matter, light microscopy, GFAP immunodetection. Detail of the ventral horn. Immunoreactive astrocytes appear as stellate cells (*arrowheads*), whose processes do not show any preferential orientation. More astroglial perikarya can be observed than in the dorsal horn. Motoneuron cell bodies (*M*), appear as unstained areas. ×530.



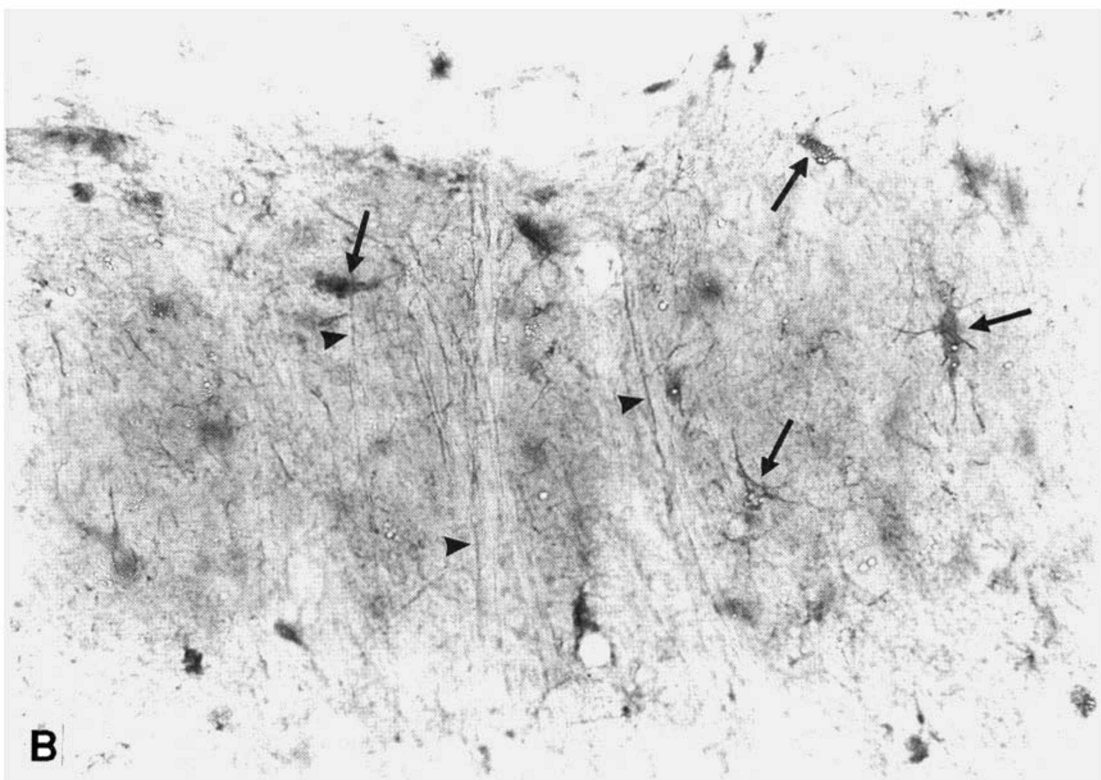


FIG. 1-6. (**A**) Rat spinal cord white matter, light microscopy, S-100 protein immunodetection. Radial glia is immunostained, but the processes cannot be observed in their entire length. The perikarya are fully impregnated as well as the main processes (*arrowheads*) *GM*, gray matter; *WM*, white matter. ×250. (**B**) Rat spinal cord gray matter, light microscopy, S-100 protein immunodetection. High magnification of the most superficial layers of the dorsal horn. Perikarya of protoplasmic astrocytes (*arrows*), are diffusely immunostained. Immunoreactive astroglial thin processes (*arrowheads*), appear preferentially radially oriented. ×530.

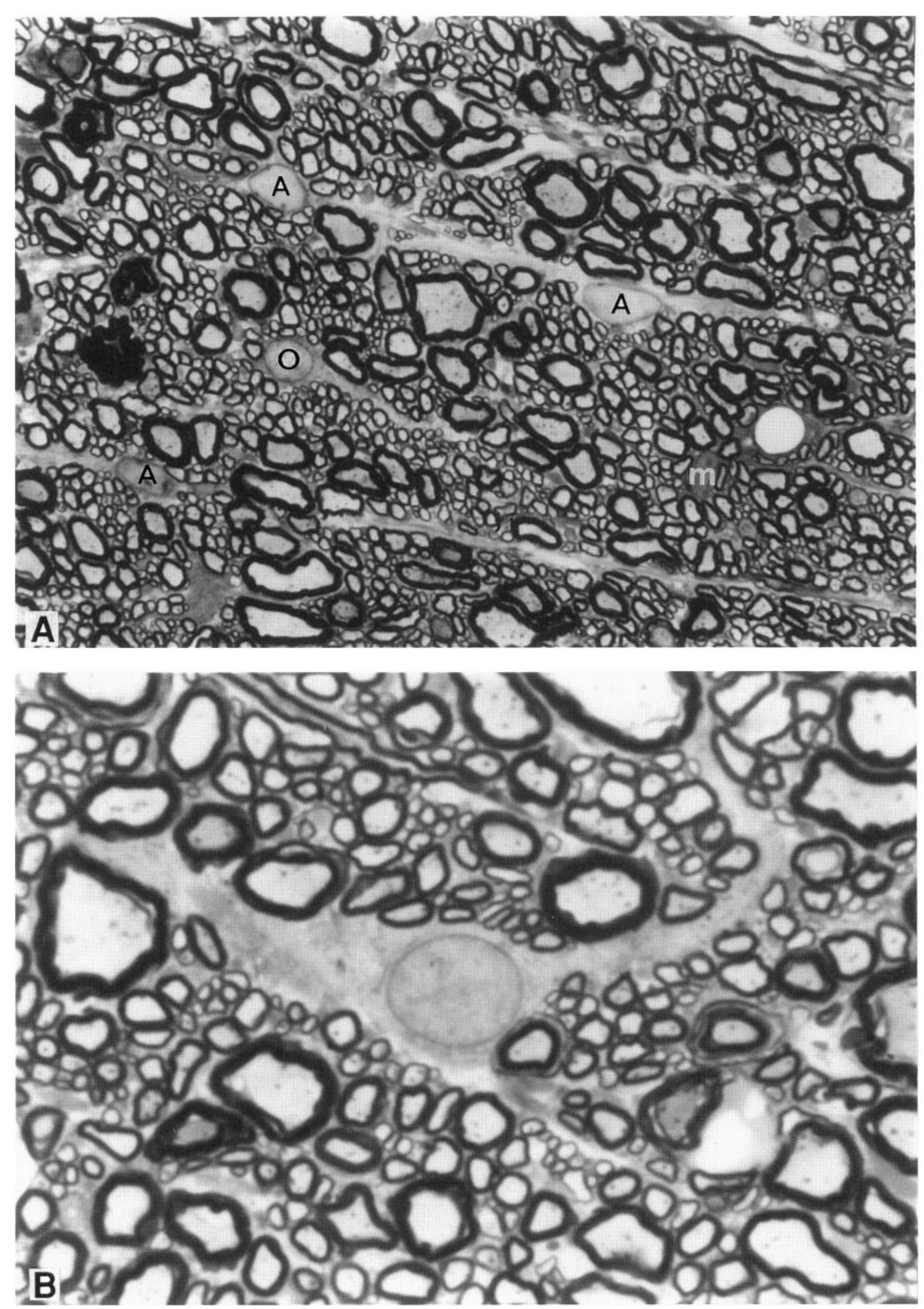


FIG. 1-7. (**A**) Rat spinal cord white matter, light microscopy, 1 μm section toluidine blue stain. The nuclei of three radial astrocytes (*A*) are surrounded by light strands of radial glia cytoplasm, delineated by the dark myelinated fibers. *O*, oligodendrocyte; *m*, microglia. ×1250. (**B**) Rat spinal cord white matter, light microscopy, 1 μm section toluidine blue stain. High magnification illustrating a fibrous stellate-shaped astrocyte: note the characteristic thin perinuclear rim of chromatin. ×3750.

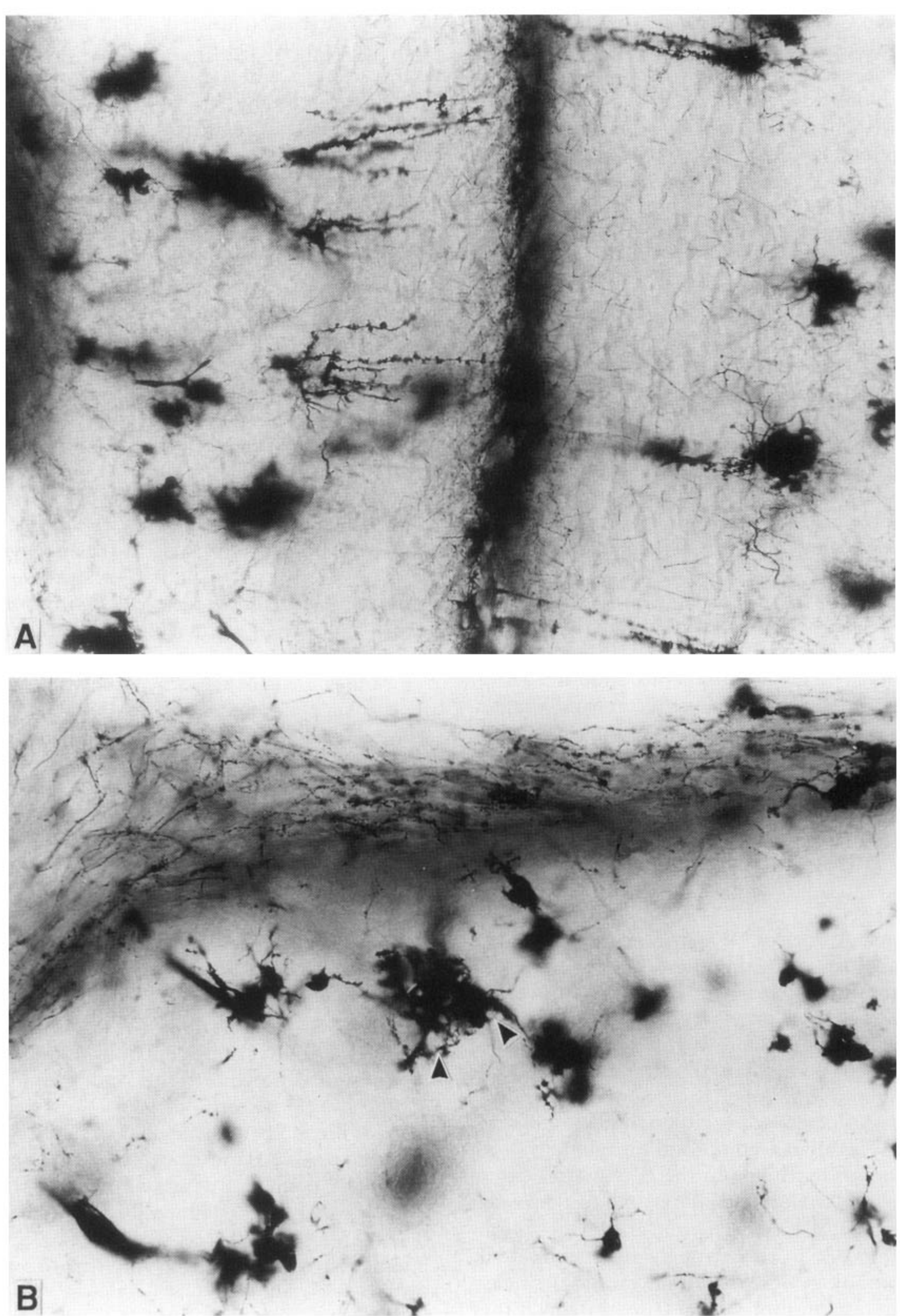


FIG. 1-8 (**A**) Rat cerebellar cortex, light microscopy, Golgi-Rio Hortega impregnation. Bergmann glial cells (Golgi epithelial cells), exhibit long thin spinous processes abutting onto the pia mater. ×250. (**B**) Rat cerebellar cortex, light microscopy, Golgi-Rio Hortega impregnation. Velate astrocyte with sheet-like appendages (*arrowheads*). ×500.

with long, thin poorly ramified processes (Figure 1-2B). These processes are generally smooth surfaced, and extend over long distances, usually without reaching the pia mater. They sometimes get in contact with blood vessels on which they abut through an expanded endfoot.

After immunocytochemical detection of GFAP, fibrous astrocytes of the white matter appear as stellate cells, whose perikaryon is incompletely stained, and whose processes appear knotted and irregular in shape and caliber (Figure 1-5A).

After immunodetection of S-100 protein (Figure 1-6A), the perikarya are better impregnated than with GFAP. Conversely, the processes appear more fragmented, with a great number of very thin profiles that one can hardly trace back to the parent perikaryon.

On semithin sections, fibrous astrocytes of the white matter can be differentiated from radial ones on the regular spherical or slightly oval shape of their nuclei, which is in the center of a light staining space that radiates in branching fashion in all directions (Figure 1-7B).

Fibrous astrocytes of the spinal cord white matter are in all respects similar to those described by Sasaki and colleagues (1989), and by other authors in other regions of the CNS: corpus callosum (Mori and Leblond, 1969), optic nerve (Peters and Vaughn, 1967). In the cerebral cortex, and specifically the subcortical white matter, impregnation with the gold chloride sublimate method of Ramón y Cajal discloses cells whose morphology is in many respects similar to that evidenced by GFAP immunocytochemistry in the spinal cord (compare Figures 1-4B and 5A).

Protoplasmic Astrocytes. When impregnated by the Golgi-Rio Hortega method, the astrocytes of the gray matter of the spinal cord appear as bushy cells with numerous short processes radiating in all directions. These processes are highly ramified and of variable caliber, contrasting with those of the fibrous astrocytes of the white matter. Some cells whose perikarya are located across the white/gray mattter border may exhibit a composite morphology, with those processes extending in the gray matter exhibiting a protoplasmic morphology, while those that extend through the white matter are of the fibrous type (Figure 1-2C). This strongly suggests that the actual morphology of astrocytes is indeed the reflection of the organization of the tissue in which they are located than of any other intrinsic characteristics (Ramón y Cajal, 1909).

After detection of GFAP, the astrocytes of the spinal cord gray matter appear as small cells with many short processes radiating from a perikaryon which is most often incompletely delineated by GFAP immunoreactive structures (Figure 1-5B,C). The nucleus is always unstained. The processes have a rather smooth outline, and are rarely branched.

After detection of S-100 protein, the perikaryon of astrocytes, including the nucleus, is delicately stained, together with the proximal part of the processes, which appear numerous, of irregular caliber, with an irregular outline and a few branchings. Processes of small caliber are also delicately stained in some regions of the gray matter, such as the superficial part of the dorsal horn (Figure 1-6B). In such instances, those processes do not appear to be in continuity with impregnated perikarya.

On semithin sections stained with toluidine blue, the nuclei of astrocytes appear as regularly spherical or ovoid, with a smooth outline and a regular condensation of chromatin along the nuclear envelope (Figure 1-9A,B). The perinuclear cytoplasm appears light on the background of the neuropile. The processes are not clearly visible at variance with the astrocytes of the white matter, whose processes are more clearly delineated by neighboring myelinated axons and densely stained oligondendrocytes (Figure 1-7A).

In other areas of the central nervous system gray matter, such as cerebral cortex, hippocampus, striatum, cerebellum, the morphology of astrocytes appears basically the same, whether it is evidenced by *in toto* impregnation with metallic deposits (Figure 1-3B,C), or by detection of a specific epitope or finally on a semithin section. There may be slight variations, for instance, in the abundance of GFAP, which is usually greater in hippocampus than elsewhere, or in the shape and size of nuclei, or in the extension and branching of processes (Figure 1-3C). What is evident is that astrocytes adapt their shape and size to the architecture of the tissue, in order to constitute a tridimensional net whose mesh size is adapted to the various functions subserved by the neurons of different regions and their companion glial cells. Specifically, astrocytes of the granular layer of the cerebellum are characterized by sheetlike appendages (velate astrocytes), which extend between the perikarya of granule neurons (Figure 1-8B). Whether their tridimensional architecture is the result of repellant forces (Distler et al., 1991), or interaction with neurons is still a matter of debate.

Ultrastructure of Astrocytes

Ultrastructural examination of all types of astrocytes in the spinal cord and in other regions of the central

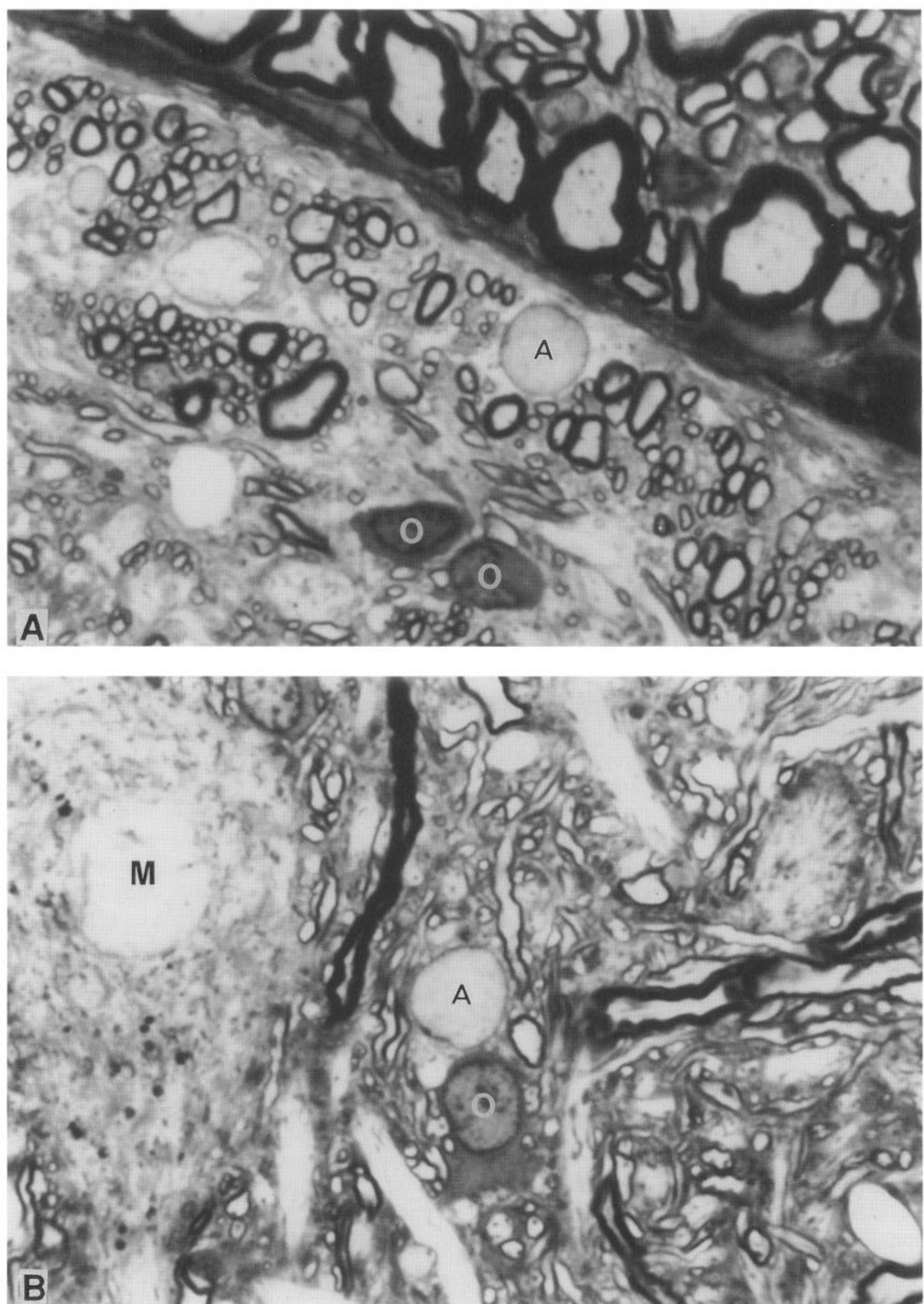


FIG. 1-9. (**A**) Rat spinal cord gray matter, light microscopy, 1 μm section toluidine blue stain. Detail of the dorsal horn showing the perikaryon of a subpial protoplasmic astrocyte (*A*) *O*, oligodendrocyte. ×2500. (**B**) Rat spinal cord gray matter, light microscopy, toluidine blue stain. Semithin section showing a protoplasmic astrocyte (*A*), and an oligodendrocyte (*O*), in the ventral horn. Note the contrast between the staining of astrocyte and oligodendrocyte cytoplasms and chromatins: the former is characterized by a clear cytoplasm and a thin perinuclear rim of chromatin, the latter by a denser and darker cytoplasm and a mottled heterochromatin. *M*, motoneuron. ×2500.

nervous system demonstrates that they share a number of characteristics, and that the rare differences encountered are quantitative rather than qualitative.

As already seen on semithin sections, the nucleus is usually regular in shape, in rare instances with small indentations. With aldehyde fixations, the nucleoplasm appears finely granular (Figure 1-10A), with a distinct condensation of chromatin along the nuclear envelope, which exhibit numerous pores (Figure 1-10A), (Mori and Leblond, 1969; Peters et al., 1976). The ground cytoplasm of the perikaryon is less dense than that of neighboring neurons and oligodendrocytes, and is poor in free ribosomes. The cisternae of rough endoplasmic reticulum are usually short with well-delineated membranes on a light background, and a content that appears denser than the ground cytoplasm. The Golgi region is not extensive, but well delineated in a juxtanuclear position. Mitochondria appear frequently less dense than those of neurons. The most specific organelle is the intermediate gliofilament, 8 to 10 nm in diameter, organized in bundles, whose quantity varies widely from one astrocyte to the other. As a rule, they are much more abundant in fibrous astrocytes of the white matter (Figure 1-11B), and much more abundant in the processes than in the perinuclear cytoplasm. They are specifically decorated with a peroxidase deposit when reacted with anti-GFAP antibody (Figure 1-11A, D). The cytoplasmic contour of astrocytes is extremely irregular; their processes insinuate between neuronal and other glial profiles, and, for instance, most often completely insulate neuronal perikarya from microglial satellites.

Processes emanating from the perikaryon are of variable caliber, usually larger in fibrous astrocytes and even larger in radial astrocytes such as those of the spinal cord white matter or Golgi epithelial cells of the cerebellum (Figure 1-10B). They contain, besides gliofilaments, isolated microtubules, that appear more concentrated in their initial segment, close to the perikaryon (Figure 1-10C). Membrane specializations of astrocytes are of two types: gap junctions involve most often two astrocytes (Figures 1-10D and 1-12A), and more rarely an astrocyte and an oligodendrocyte, or the outer leaflet of a myelinated fiber (Mugnaini, 1982). They are of variable surface and are found on perikarya as well as on processes, with a particular concentration on subpial and perivascular endfeet (Figure 1-12B). The molecular substrate of these astrocyte-astrocyte gap junctions is a protein, connexin 43, which is part of a gene family comprising a dozen protein that have a similar gene structure, and about 50 percent sequence identity at the amino acid level (for review see Dermietzel and Spray, 1993). It has been shown recently that the abundance of gap junctions between astrocytes, that is the expression of connexin 43, displays topographic differences (Dermietzel et al., 1989; Yamamoto et al., 1990). For instance, connexin 43 is more abundant in the hypothalamus than in the striatum (Batter et al., 1992). At variance, gap junctions between oligodendrocytes involve another connexin, connexin 32. Thus, astrocyte-oligodendrocyte gap junctions would eventually be heterotypic junctions, with properties different from those of either astrocytes or oligodendrocytes (for review see Dermietzel and Spray, 1993).

The other characteristic feature of astrocyte membranes, as seen with freeze-fracture, is the so-called orthogonal assembly, described first by Dermietzel (1974), on astrocytes and later found by Privat (1977), on ependymocytes. These assemblies, found only on astrocytes and related cell types (Mugnaini, 1982), consist of paracrystallin arrays of 7-nm subunits, appearing as particles on the P-face and small pits on the E-face. They are especially abundant on astrocyte surfaces apposed onto mesenchymal tissues, that is, the subpial and perivascular endfeet (Figure 1-12B). Whatever the region of the central nervous system, ultrastructural examination reveals that astrocyte processes fill all the spaces between neurons and other glial cells, thus substantiating their role in insulating them from each other. This insulating capacity may take various forms, according to the specific characteristics of neural circuits. We have studied in detail the glial and neuronal environment of a specialized portion of monoaminergic fibers present in the dorsal horn of the cord. It has been demonstrated over the last decade (Maxwell et al., 1983, 1985; Marlier et al., 1991; Rajaofetra et al., 1992; Ridet et al., 1992, 1993), that most of the monoaminergic axonal boutons in the spinal cord dorsal horn did not contribute classical synapses. Reconstruction from serial sections evidenced the absence of postsynaptic densities facing a majority of these axonal boutons (Ridet et al., 1993). Analysis of tissular environment of these varicosities has shown that the proportion of astroglial profiles was higher around serotonergic and noradrenergic varicosities devoid of synaptic specializations. Moreover, the length of the contact between nonsynaptic varicosities and astrocytes was twice as long as that between synaptic varicosities and astrocytes (Ridet et al., 1993). The specialized type of communication corresponding to this morphological organization, the so-called "volume transmission" (Fuxe and Agnati, 1991), could thus involve specialized astrocytes. It is highly likely that careful tridimensional reconstruction, associated with the de-

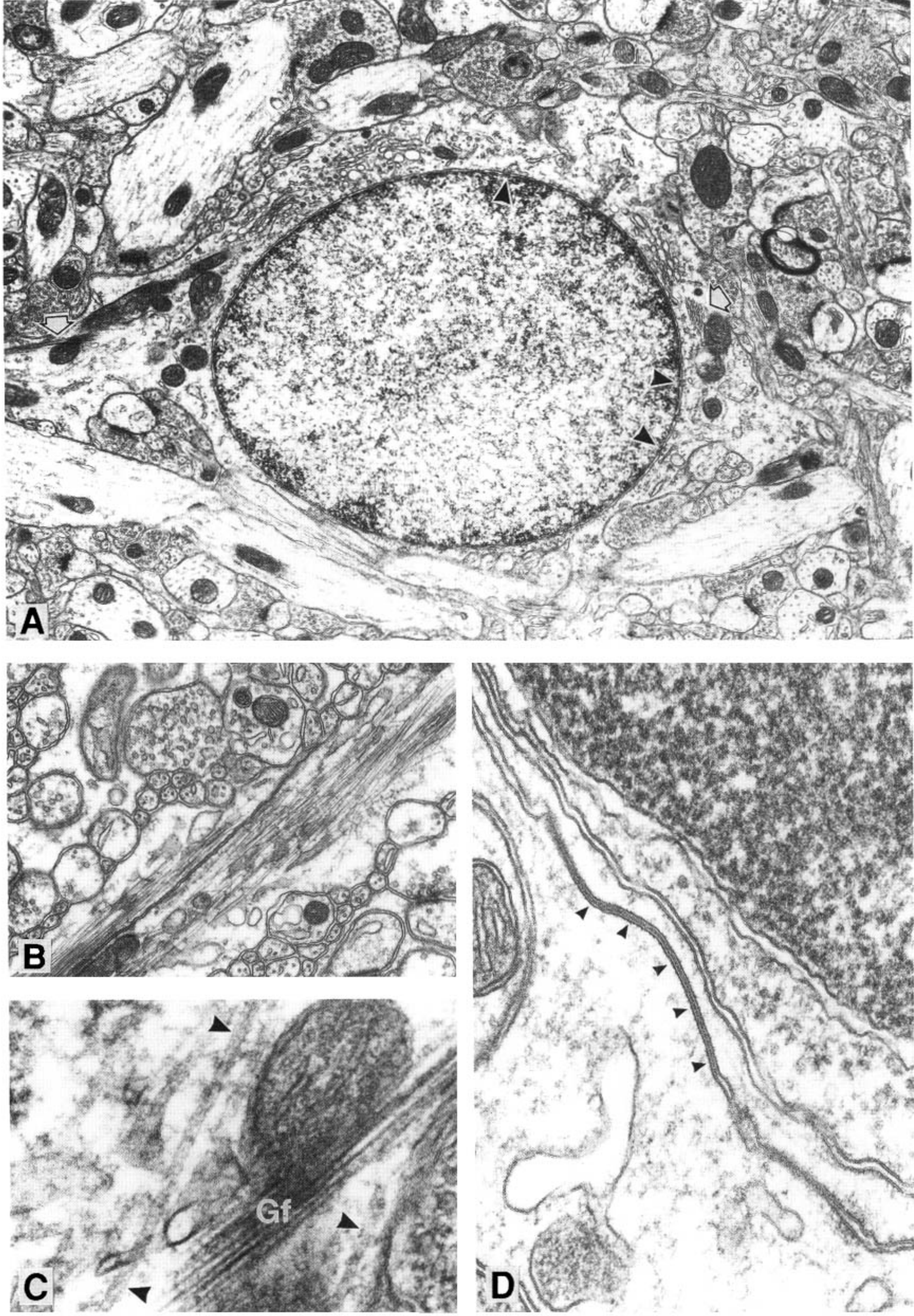


FIG. 1-10. **(A)** Rat cerebral cortex gray matter, electron microscopy. Detail of a protoplasmic astrocyte, exemplifying the light cytoplasm with an irregular outline, the light nucleus with clumped chromatin along the nuclear envelope. *Arrowheads* point to nuclear pores, *arrows* to gliofilaments. ×13,600. **(B)** Rat cerebellar molecular layer, electron microscopy. Detail of astroglial processes of Golgi epithelial cells showing bundles of gliofilaments. ×20,000. **(C)** Rat cerebral cortex gray matter, electron microscopy. High magnification of astroglial cytoplasm showing isolated microtubules (*arrowheads*), and a bundle of gliofilaments (*Gf*) ×88,900. **(D)** Rat cerebellar granular layer, electron microscopy. High magnification of a gap junction *(arrowheads)*, between a large and a thin strip of astroglial processes. The heptameric structure of the junction is clearly apparent. ×76,700.

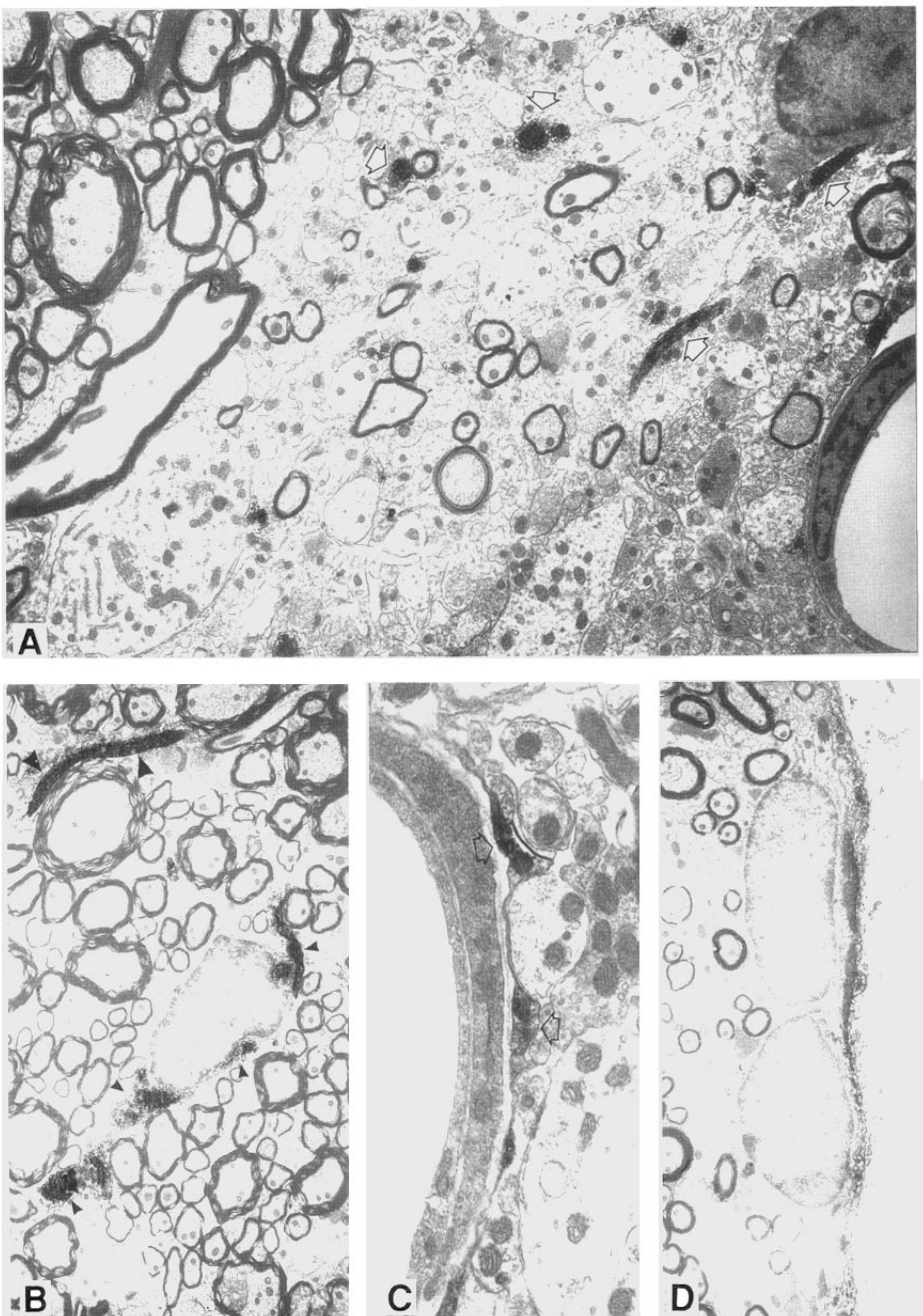


FIG. 1-11. (A) Rat spinal cord gray matter, electron microscopy, GFAP immunodetection. Pattern of distribution of immunoreactive gliofilaments (*empty arrows*), in the dorsal horn. ×6000. (B) Rat spinal cord white matter, electron microscopy, GFAP immunodetection. Immunoreactivity is concentrated in the cell body (*small arrowheads*), of a fibrous astrocyte and in a large process (*large arrowheads*) ×4200. (C) Rat spinal cord gray matter, electron microscopy, GFAP immunodetection. Immunoreactive perivascular astroglial processes are pointed with *empty arrows*. ×18,400. (D) Rat spinal cord gray matter, electron microscopy, GFAP immunodetection. Subpial astrocytes, constituting the glia limitans at the surface of the dorsal horn, which is characterized by multiple thin lamellar processes, exhibiting immunoreactive gliofilaments. ×4400.

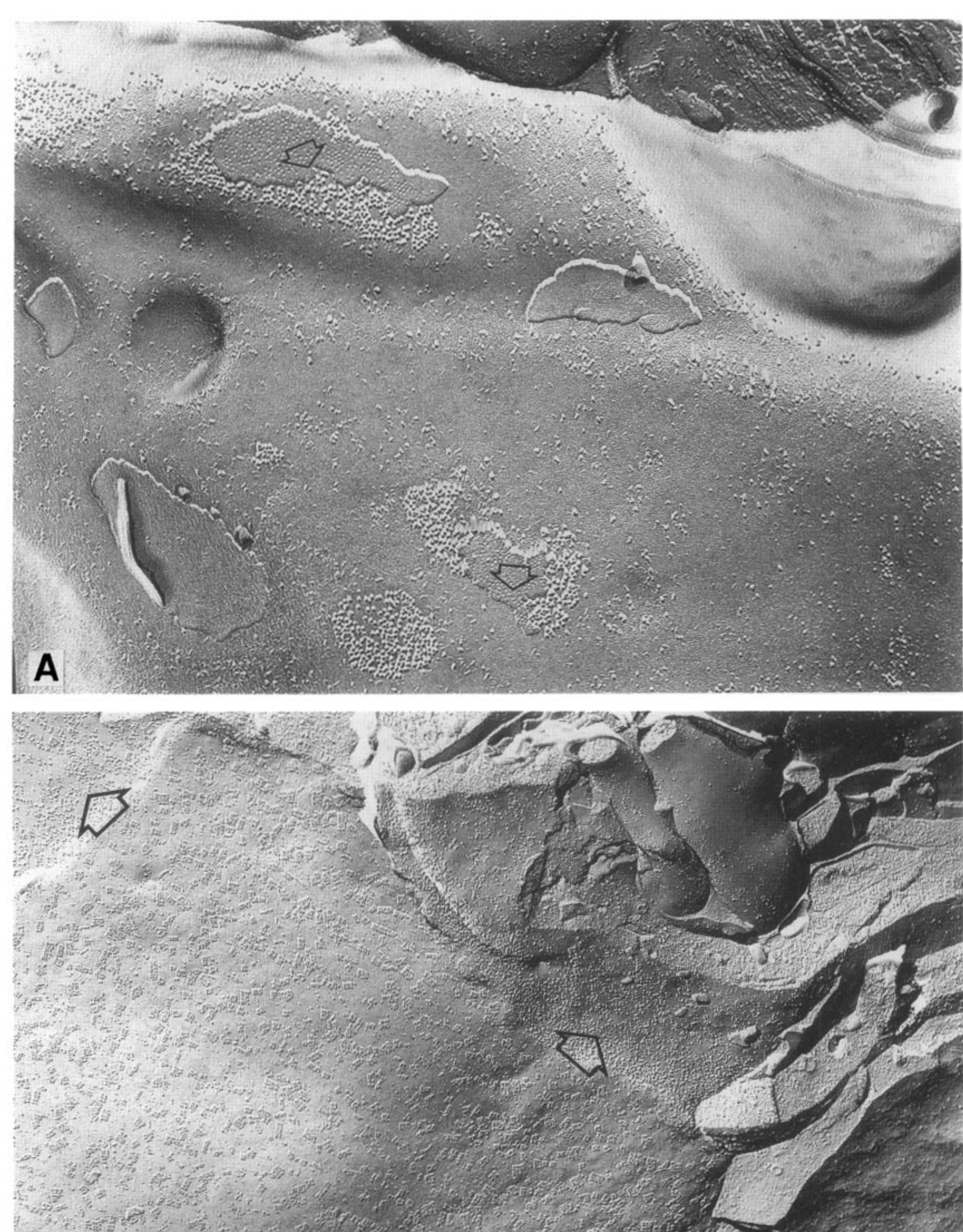


FIG. 1-12. **(A)** Rat corpus callosum, freeze-fracture replica. Three macular gap junctions in the membrane of a fibrous astrocyte. On two of them (*arrows*), patches of attached membranes exhibit complementary pits. ×80,000. **(B)** Mouse spinal cord white matter, freeze-fracture replica. A subpial endfoot exhibits, on the surface contacting the basal lamina, typical orthogonal assemblies. Notice that the lateral membranes (*arrows*), are almost devoid of assemblies. *E*, E-face; *P*, P-face. ×60,000.

tection of specific receptors or uptake systems, would reveal exquisite differentiations of astrocytes in almost any region of the central nervous system.

CONCLUSIONS

The study of the morphology of astrocytes tells us two things:

1. There is a great diversity of shapes and sizes of astrocytes and their processes throughout the nervous system. Interestingly, the spinal cord provides a unique example of a region of the central nervous system where all astrocytes subtypes are represented: radial glial, fibrous, and protoplasmic astrocytes. This variety is in accordance with the organization of the different tissue compartments of the cord: the radial arrangement accommodates the funicular organization of descending and ascending axons of the white matter. The few fibrous astrocytes, found mainly in the ventral funiculi, are likely to accommodate the projection of motoneuron axons. Protoplasmic astrocytes of the gray matter are molded upon the complex tridimensional neuropile and, as such, occupy roughly a spherical volume.
2. All these cells have in common the expression of a specific phenotype, including the synthesis of proteins such as GFAP and S-100, the presence of gliofilaments, the qualitative identity of nuclear and cytoplasmic ultrastructural characteristics, and of membrane specializations.

It is not yet totally clear whether the phenotypic diversity results exclusively from the interaction of a common genotype with the tissular environment, or whether a mosaic of phenotypes exist in the neural tube. It is most likely that the two coexist, that is, some degree of diversity is already expressed in early progenitors, a diversity that is later expanded during prenatal and postnatal ontogeny.

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2 The morphology and ultrastructure of oligodendrocytes and their functional implications

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Such minute corpuscles could not evoke any aesthetical emotions. Yet these cells, refractory to every protoplasmic staining, because of their infinite numbers, their dissemination in encephalic terrains and their strategic location next to neurons and nerve fibers, deserved, at some point, to seize the attention of neurologists and to be placed on an equal rank with the classic neuroglia. This moment has arrived!

P. d. Rio Hortega (1928)

Oligodendrocytes (OLGs) are defined as the cells that make and maintain central nervous system myelin. This definition came into being only about three decades ago, despite the fact that in 1928 Rio Hortega had clearly shown the association of OLGs with myelin and had postulated the functional similarity of OLGs to Schwann cells (Rio Hortega, 1925). The reason for this protracted uncertainty as to the function of OLGs rests on the inherent refractiveness of OLGs to stains, on the complex nature of OLG-axon interaction and on the intricate topography of the central nervous system.

Without denying recognition to Robertson (1899) and Ramón y Cajal (1913), who visualized cells that, in all probability, were OLGs, I believe that few would argue that it is Rio Hortega (1921, 1922, 1928) who deserves recognition as the discoverer of OLGs. By using innovative metal impregnation techniques based on silver carbonate (Rio Hortega, 1921, 1922) or silver nitrate—a modification of Golgi's technique (Gurr, 1962)—Rio Hortega (1928) rendered the first detailed description of the morphology of cells that he named oligodendroglia (ὀλίγοσ = few; δὲνδρον = tree; γλία = glue). An afterthought prompted Rio Hortega (1928) to change the name to *oligodendrocytes*—in recognition that they were bona fide cells. The importance of Rio Hortega's contribution goes beyond the structural characterization of OLGs; exhibiting great insight, he suggested that OLGs make the myelin sheath and thus are the Schwann cells of the central nervous system.

This chapter deals with the morphology and ultrastructure of OLGs. There are a number of textbooks (e.g., Haymaker and Adams, 1982; Peters et al., 1991) and reviews (e.g., Mugnaini and Walberg, 1964; R. Bunge, 1968; Szuchet and Stefansson, 1980; Szuchet and Dumas, 1983; Wood and Bunge, 1984) that describe these topics comprehensively. One may ask: Is there a need for more of the same? For the sake of completeness, some overlap with the above-cited references is unavoidable, but this chapter is not intended to be a historical, all-inclusive review of findings; rather, the emphasis will be on integrating current concepts and technologies in an attempt to establish a link—however tenuous—between the structure and function of OLGs. Two examples may illustrate some of the perplexities that need to be addressed. On the one hand, OLGs exhibit structural polymorphism, and heterogeneous interaction with axons: Does this imply functional heterogeneity among the myelin-making OLGs? On the other hand, morphology does not appear to be a distinguishing feature between a "perivascular" and a perineuronal OLG: Can it be assumed that they have similar functions? These are some of the issues that are discussed here; unfortunately, much of it is in the realm of speculation.

The development of techniques for isolating and maintaining OLGs in long-term culture (McCarthy and de Vellis, 1980; Szuchet et al., 1980a, 1980b; Gebicke-Härter et al., 1981; Lisak et al., 1981; Hertz et al., 1985) has spurred a strong interest in the biology of OLGs. Witness to this is the large bibliography that has accumulated. While new insights pertaining to the topics discussed here have yet to emerge, advances in the knowledge of signaling events that initiate and control myelin formation and of the mechanisms of gene expression are bound to pave the way for a functional understanding of OLG polymorphism. Paraphrasing Stensaas and Stensaas (1968b), it is axiomatic that the morphology of the various OLG subtypes is related to their respective function.

This chapter is divided into four main sections. The first section reviews current findings on the morphology and ultrastructure of OLGs and discusses them in the context of the classical work of Rio Hortega. The second section examines the functional significance of OLG polymorphism. The third section deals with *in vitro* models and their contribution to our understanding of the OLG structure-function relationship. In the fourth section, the role of the substratum, other central nervous system cell types, and trophic factors in influencing or determining the morphology of OLGs is assessed.

FROM RIO HORTEGA TO THE PRESENT: WHAT IS NEW?

Morphology

The complexity of OLG morphology must have astonished Rio Hortega, for he stated: "If OLGs would represent a type of cell with a uniform morphology as is the case for protoplasmatic or fibrous astrocytes, then a description of their morphology would be a simple matter. As is, OLGs are so polymorphic that each variant has to be characterized independently" (Rio Hortega, 1928). I have chosen to describe the morphology of OLGs as portrayed by three different techniques because each reveals distinct aspects.

Metal Impregnation. Due to a peculiar resistance to stains, OLGs were the last central nervous system cells to be discovered and characterized. Rio Hortega's success where others had failed was in large measure due to his use of silver impregnation techniques, particularly the silver nitrate method (Rio Hortega, 1928). He observed that the ease of impregnation varied among species and explored those that gave the best results (e.g., kittens or dogs); he also observed that delineation of cellular extensions was better in young animals than in old ones. Rio Hortega found OLGs to be distributed throughout the central nervous system, but in higher abundance in the white matter. He noted three kinds of dispositions: (1) alignment of closely apposed cells in rows along nerve fascicles, (2) juxtaposing neuronal somata, and (3) abutting blood vessels (see below). Accordingly, Rio Hortega classified OLGs into interfascicular, perineuronal, and perivascular. Additionally, using criteria such as size of somata, number and characteristics of cellular processes, tissue distribution, and the manner of interaction with axons, Rio Hortega (1928) grouped OLGs into four subtypes, which he numbered I through IV, recognizing nevertheless the absence of transition limits among them.

Type I OLGs (Figure 2-1A) have spherical or slightly polygonal somata (15 to 20 μm) from which abundant, tenuous processes emerge directed toward nerve fibers; they can be found in the forebrain, cerebellum, and spinal cord and are organized around blood vessels, neurons, and fiber tracts. Type II OLGs (Figure 2-1B) are observed only in white matter; they have a polygonal or cuboid shape (20 to 40 μm), have fewer and thicker processes than do type I OLGs, and have a close association with nerve fibers. Type III OLGs, with three to four processes emanating from the somata and directed toward nerve fibers (Figure 2-1C), are localized in the cerebral and cerebellar peduncles, the medulla oblongata and the spinal cord. Finally, type IV OLGs (Figure 2-1D) occur near the entrance of nerve roots into the central nervous system and in association to large axons (Friedrich et al., 1980). They adhere directly to the nerve fibers not unlike Schwann cells (Polak, 1965).

Figure 2-1 illustrates prototypes of each class. It is clear from these photomicrographs that Rio Hortega has captured the full gamut of OLG polymorphism. It is also apparent that no sharp boundaries exist between the various subtypes. For example, the type I cell C in Figure 2-1A bears a closer resemblance to the type II cell (Figure 2-1C) than to type I cell A in Figure 2-1A. This fact has already been emphasized by Rio Hortega (1928). Nonetheless, when one compares cell A in Figure 2-1A with cell A in Figure 2-1D, the difference is impressive. The implications of this polymorphism have yet to be fully realized. One might conjecture that this structural diversity has its functional correlates, but data to support such a contention have been slow in coming. Future research should clarify this issue.

The work of Rio Hortega, confirmed at the time by Penfield (1932) and others, met with a great deal of skepticism, especially in the early days of electron microscopy. But his assertions on the role of OLGs have withstood the test of time, even though his accomplishments are scarcely appreciated even today.

Metal impregnation techniques, although capable of providing detailed information on cell morphology when used by gifted hands, often produce erratic results and therefore have not been fully explored. Nevertheless, Stensaas and Stensaas (1968a) applied the method of Golgi-Rio Hortega to examine OLGs in toad spinal cord and detected a spectrum of OLG morphologies that bore a resemblance to the subtypes in the mammalian central nervous system. Figure 2-2 depicts the range of morphologies seen between two of the prototypes, type I (Figure 2-2A)

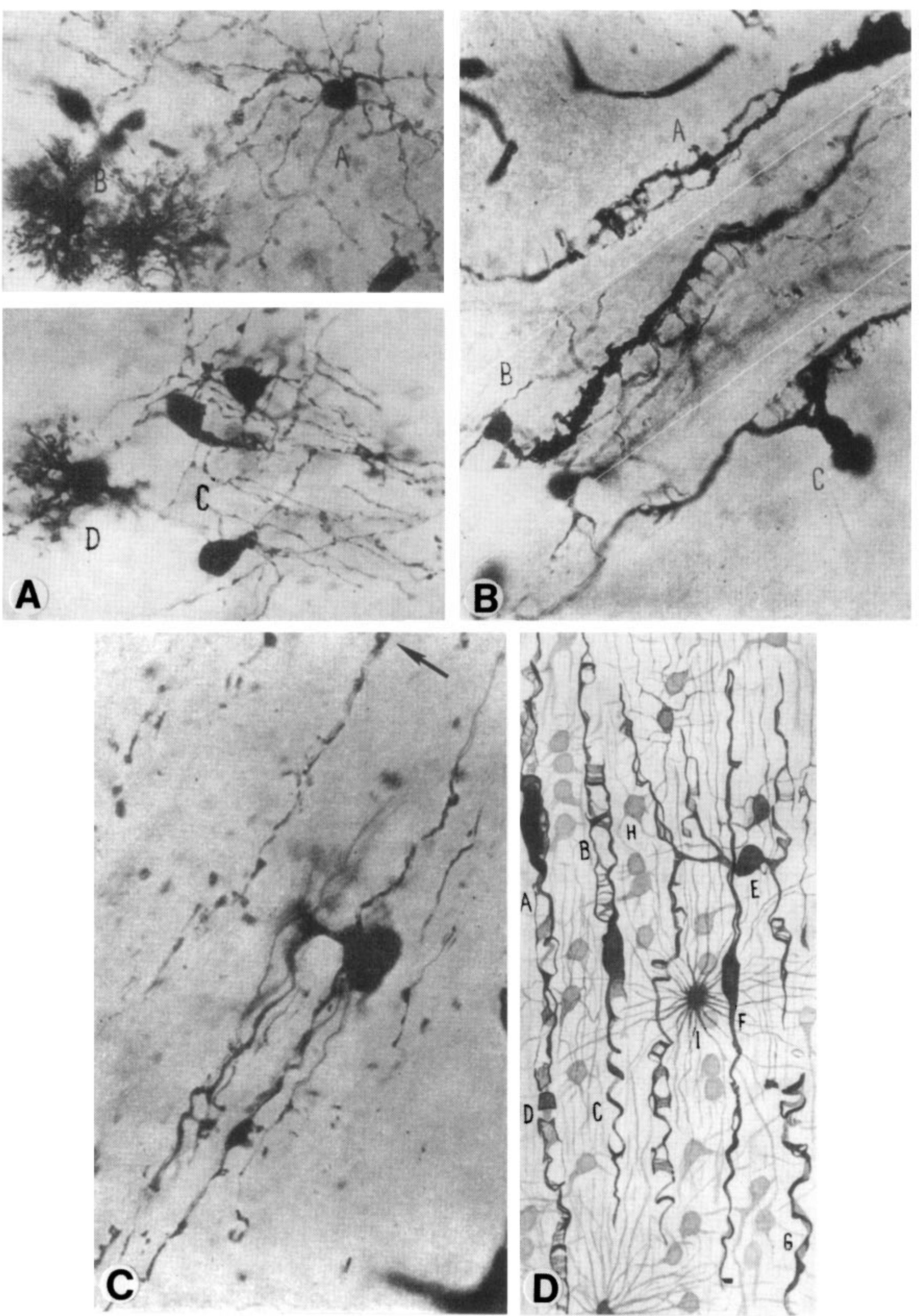


FIG. 2-1. Oligodendrocyte polymorphism. (**A**) Photomicrograph of type I oligodendrocytes from canine cerebral white matter. (*A*) Oligodendrocyte with processes irradiating from cell body; (*B*, *D*) dwarf astrocytes; (*C*) oligodendrocytes with intricate processes. (**B**) Photomicrograph of type III oligodendrocytes from cerebral penduncles. (*B*) Monopolar and (*C*) bipolar cells. (**C**) Photomicrograph of type II oligodendrocyte from cerebellar white matter with parallel processes. Arrow points to ringlike structure; cf. Figure 2-3. (**D**) Oligodendrocytes from feline medullar white matter. (*A,B,F*) Type IV with reticula and rings; (*C,G*) with helices; (*E*) type III; (*H*) type I. [From Rio Hortega (1928), with permission]

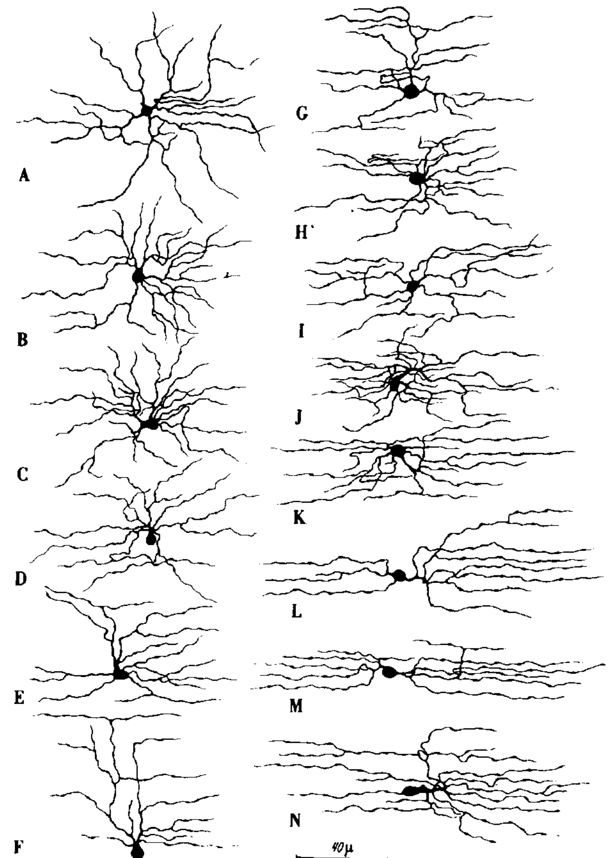


FIG. 2-2. Oligodendrocytes of types I and II. (*A–N*) arranged to show the variation in the orientation of the long, thin processes. Parallel processes are more common for cells located in the white matter but can also be seen in the peripheral plexus. [From Stensaas and Stensaas (1968), with permission.]

and type II (Figure 2-2B). These authors questioned the existence of morphological continuity between all subtypes because no transitional form was observed between types I and II on the one hand, and types III and IV on the other. The employment of a modified version of the metal impregnation technique led to the claim for the existence of a fifth type of OLG (Klatzo, 1952) and to the definition of an elaborate topographical map of OLG-neuronal interconnections (Ogawa et al., 1985).

Visualization of Oligodendrocytes by Intracellular Injection of Dyes. Butt and Ransom (1989) injected optic nerve cells with Lucifer yellow (or horseradish peroxidase). Figure 2-3A illustrates the appearance of cells presumed to be OLGs [later confirmed by electron microscopy (Ransom et al., 1991)]. The authors describe these cells as having 20 to 30 parallel processes that often terminate in loops (arrow in Figure 2-3A) and are connected to the cell somata by a number of fine processes 15 to 30 μm in length. The longitudinal processes are interpreted as corresponding to the tongues of the myelin sheath. This can be better appreciated from a camara lucida drawing (Figure 2-3B). The cells shown in Figure 2-3A and B exhibit a great resemblance to type II OLGs (Figure 2-1C). Notice that processes in Figure 2-1C also terminate in loops (see the arrow). It seems that intracellular injection provides a picture with higher fidelity than metal impregnation. The limitation of the latter technique was stressed by Rio Hortega, who commented on the difficulties of achieving complete penetration. Thus it is likely that the small number of processes emanating from the cell shown in Figure 2-1C is due to poor impregnation. The dye-injection technique also affords the possibility of assessing the number of axons myelinated by a single OLG and the length of a paranode.

That Butt and Ransom (1989) observed only OLGs with a type II morphology may be due to the restricted central nervous system area examined. Given that this methodology appears to provide a clear depiction of cell somata and their processes, it would be instructive to extend such studies to other areas of the central nervous system in order to gather information on the other OLG subtypes. The major drawback to this methodology is that cells have to be independently identified.

Use of Immunocytochemistry to Investigate Oligodendrocyte Morphology. A number of antigenic markers have been exploited to investigate OLGs *in situ* (review by Sternberger, 1984). While the initial work may not have been undertaken with cell morphology as a primary goal, these studies may serve that purpose. For instance, the morphology of myelinating OLGs can be brought to light with an antimyelin basic protein antibody (Ab) or an antiproteolipid protein Ab, as illustrated in Figures 3, 4, and 5 in Sternberger's Chapter 4 in *Oligodendroglia* (1984). But these Abs are of little use in revealing OLG morphology in the mature brain. Thus antigen (Ag) concentration and Ab penetration are two factors that may present practical limitations. Nevertheless, the power of immunological methods resides in the functional information they provide while eliminating the uncertainty of cell identification. Two studies using monoclonal Abs (mAbs) may serve to illustrate the usefulness of the immunocytochemical approach. One concerns the staining of adult spinal cord with the mAb Rip—an OLG-specific mAb (Friedman et al., 1989)—because it yields Golgi-like patterns (Figure 2-4). The authors identified OLG types I to III but were unable to localize type IV OLGs and suggested that they may not be present in the spinal cord. This would be contrary to the finding of Re-

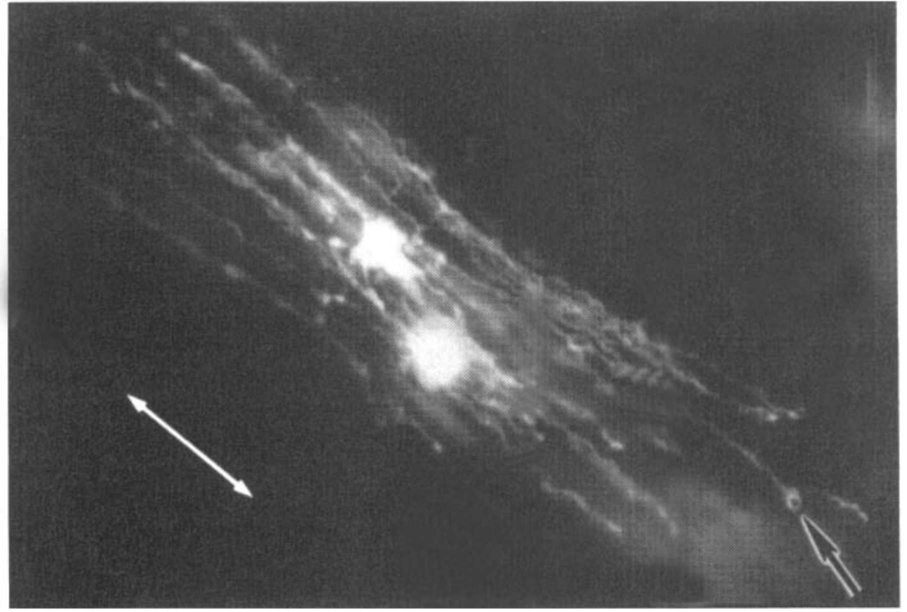
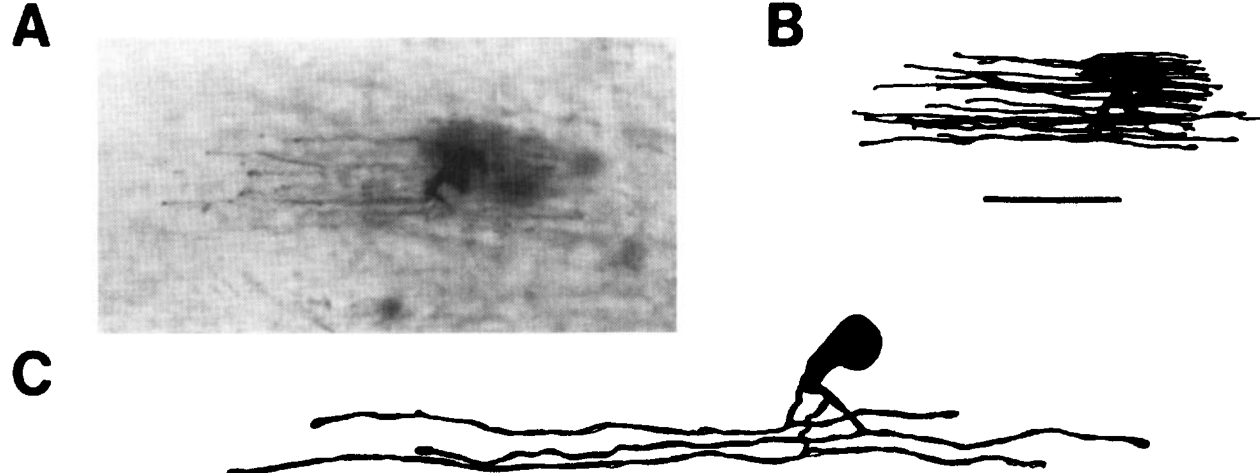


FIG. 2-3. (A) Lucifer yellow-filled oligodendrocytes in the rat optic nerve. These cells have 20 to 30 parallel processes 150 to 200 μm in length exclusively oriented along the long axis of the nerve and connected to the cell body by short thin branches. *Arrow* points to ringlike structures presumed to correspond to paranodal loops. (**B**). Interpretation of the dye-filled image of a presumed oligodendrocyte. **A**, Photomicrograph of horseradish peroxidase-filled oligodendrocytes. Scale bar = 100 μm. **B**, Camera lucida drawing of cell in *A*. **C**, Camera lucida drawing of *A* at higher power (×1250); the cell body and three selected longitudinal processes have been drawn. [From Butt, and Ransom (1989), with permission.]



mahl and Hildebrand (1990), who noted that type IV OLGs were restricted to the spinal cord. An alternative explanation could be that type IV OLGs do not carry Rip. Should subtype-specific Ags be discovered, it would not only facilitate enormously the task of identifying the different subclasses but could be instrumental in defining functional properties.

The other example of the use of an mAb to delineate OLG morphology and define the connections between OLGs and other central nervous system components is illustrated in Figure 2-5. mAb Otex1, generated against OLG plasmalemma components (Mori de Moro et al., 1990; D. Arvanitis, G. Mori de Moro, and S. Szuchet, unpublished observations), brings out OLG soma (see the arrowhead in Figure 2-5) and processes in the basis pontis of an adult human brain (Figure 2-5A). The staining is specific; neurons remain clear of reaction products (see the arrows in Figure 2-5A). Intense staining of a group of OLGs and their intricate network of long processes in the cerebellar white matter of a 2-month-old infant is shown in Figure 2-5B. The cell in Figure 2-5A has type I characteristics, whereas in Figure 2-5B types I and II are intermixed. Figure 2-5C depicts an OLG abutting a neuron—a perineuronal satellite. As the number of mAbs directed at OLG-specific Ags increases, and as the newer methods (e.g., confocal microscopy and digital imaging) become accessible for general use, OLG subtypes might be distinguished by their patterns of staining.

The evidence is strong that OLGs constitute a polymorphic family of cells, most of which have a common functional task, the assembly of myelin. The pursuit of other elements that might unite or diversify them remains an interesting proposition. This polymorphism is not restricted to the mam-

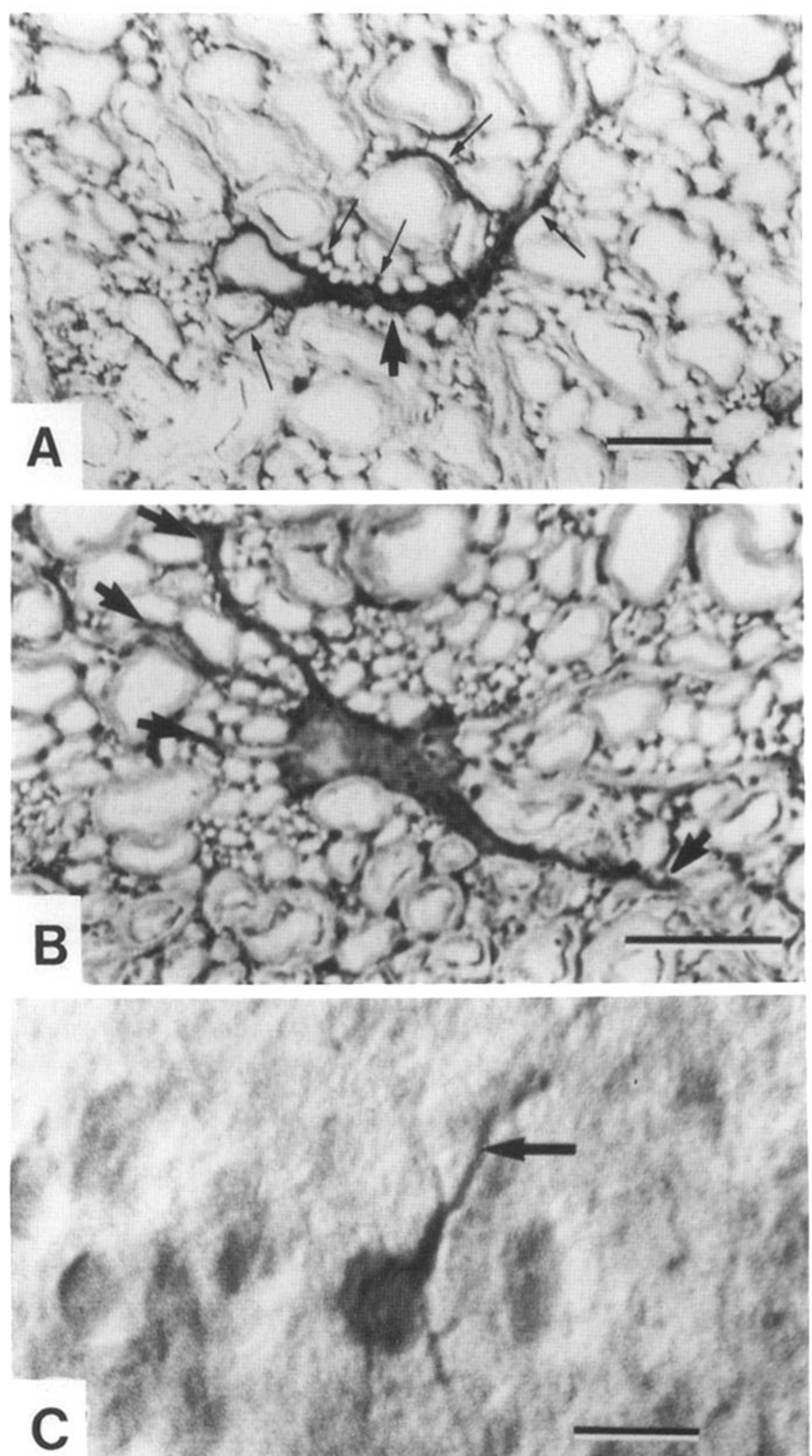


FIG. 2-4. Staining tissue sections with mAb Rip. Rip stains oligodendrocyte somata and their processes in a Golgi-like manner. Scale bar = 10 μm. (A) Oligodendrocytes with long and stout trunk processes that have fine branches. These cells resemble type II oligodendrocytes. (B) Oligodendrocyte with several stout processes (*arrows*) extending directly from the cell body. This cell resembles the type III cells of Rio Hortega. (C) Satellite oligodendrocyte similar to Rio Hortega type I oligodendrocytes.

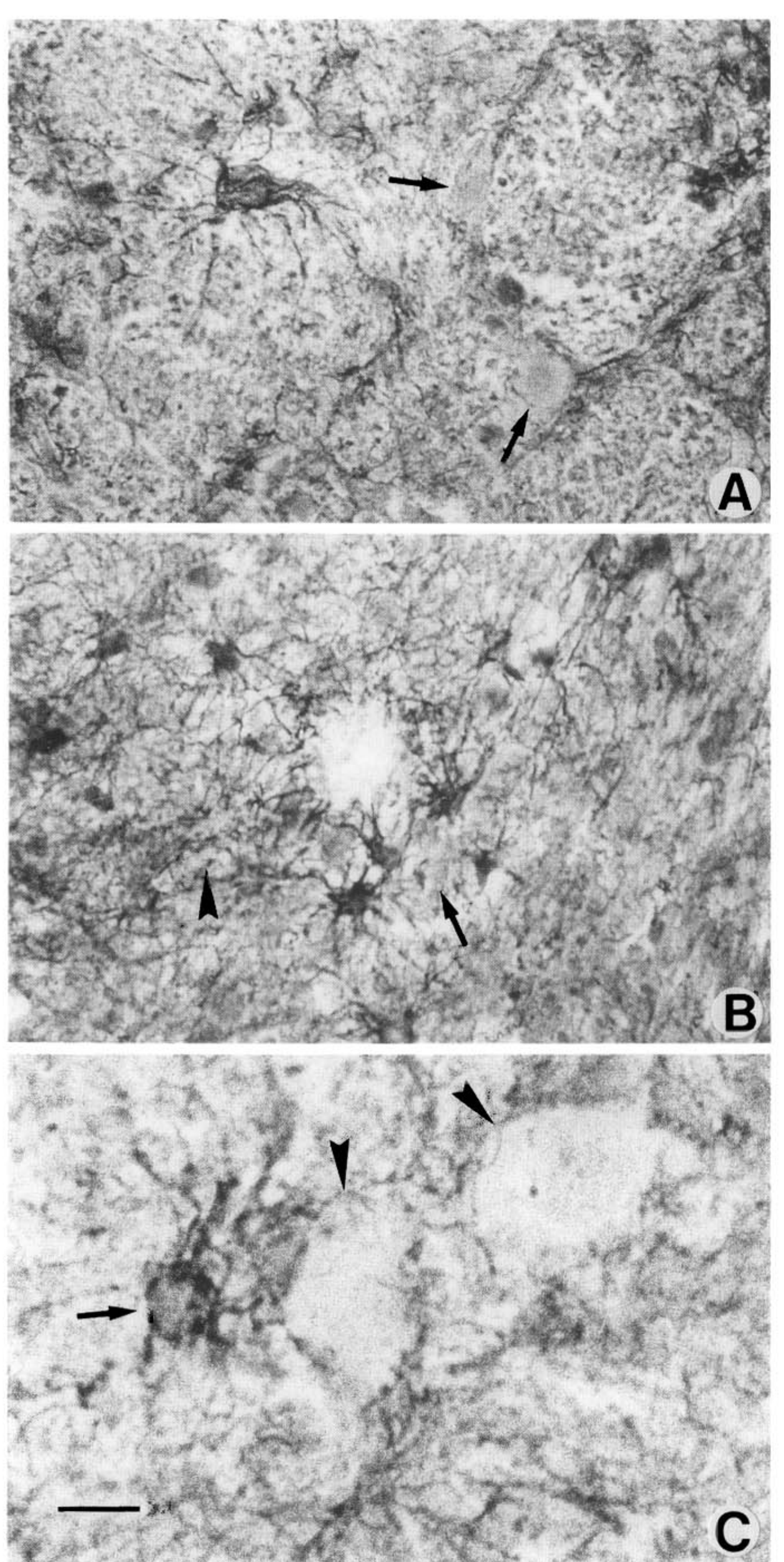


FIG. 2-5. Staining of tissue sections with mAb Otex1. The mAb was generated by injecting mice with live oligodendrocytes. Hybridomas were screened against an oligodendrocyte Triton X-100 extract; only clones that were positive for oligodendrocytes but negative for fibroblasts, astrocytes, and microglial cells were selected, expanded, and cloned. (**A**) Basis pontis from an adult brain. Oligodendrocyte showing strong staining of soma and processes with mAb Otex1. This cell fits the description of a type I oligodendrocyte (*arrowhead*). Neurons are negative (*arrows*). (**B**) Cerebellar white matter of infant. Group of oligodendrocytes organized in rows (*arrow*). Cell somata and process are strongly positive. Note extensive network of processes strongly positive (*arrowhead*). (**C**) Satellite oligodendrocytes in the basis pontis from adult brain. Positive oligodendrocyte (*arrow*) abutting an unstained neuron (*arrowhead*). Arrowhead points to a second neuron. Bar (in *C*) = 32 μm in Figures **A** and **B**; 15 μm in Figure **C**. [D. Arvanitis, G. Mori de Moro, and S. Szuchet, unpublished data.]

malian central nervous system but is found across species. Ramón y Cajal believed that "In the form you meet the whole past and present functioning" (Young, 1991). Expressed differently, structure and function are integrated; one (structure) is a reflection of the other (function). The challenge then is to decipher the meaning of the structural entities.

Ultrastructure

A detailed description of the fine structure of OLGs can be found in textbooks (e.g., Peters et al., 1976, 1991; Haymaker and Adams, 1982), in reviews (e.g., Szuchet and Dumas, 1983; Mugnaini and Walberg, 1964; R. Bunge, 1968; Wood and Bunge, 1984), and in myriad original articles. Thus only a brief summary of salient features is presented here, placing emphasis on aspects of ultrastructure that have not been stressed before.

Viewed by electron microscopy, OLGs appear as moderately dense cells relative to astrocytes. No outstanding differences in the fine structure among OLGs localized in different areas of the central nervous system have been noticed. The nucleus is round or oval and is usually eccentrically located, leaving a large mass of cytoplasm at one pole of the cell. However, sections where the nucleus is surrounded by a thin rim of cytoplasm are also seen. This seems to be the case for interfascicular OLGs. The nuclear content exhibits a slight clumping of chromatin that appears as a rim beneath the nuclear envelope. Stensaas and Stensaas (1968a) have characterized the nuclei of the three types of neuroglial cells from toad spinal cord, that is, astrocytes, OLGs, and microglia. Even at the light microscopic level, the nucleus of each cell type has a distinct appearance. The cytoplasm of OLGs is rich in ribosomes, either free or associated with the endoplasmic reticulum, which is believed to contribute to its electron density. The Golgi apparatus is well developed. Two features—apart from size and electron density—distinguish OLGs from astrocytes and serve to differentiate these two central nervous system cell types. OLGs neither have glycogen granules nor bundles of specific intermediate filaments, such as glial fibrillary acidic protein, which are characteristic of astrocytes. OLGs contain inclusion bodies of various types (Mugnaini and Walberg, 1964).

Ultrastructural Diversity. Electron microscopy reveals another aspect of OLG diversity whose direct relationship to the subtypes described by Rio Hortega have yet to be clarified. Cells identified as OLGs by criteria such as continuity with the outer tongue of the myelin sheath, or characteristic features of intracellular organelles, including the presence of microtubules, were observed to vary in size and cytoplasmic and nuclear densities. Kruger and Maxwell (1966) set out to search for Rio Hortega's polymorphism; instead they found a wide range of cytoplasmic and nuclear matrix densities among interfascicular OLGs from adult rats. They observed a higher abundance of the lighter species than the darker one. All variants were associated with axons, implying no apparent functional distinction concerning myelination. Kruger and Maxwell (1966) found perineuronal OLGs to be more dense than interfascicular OLGs. OLGs in gray matter also exhibited a range of matrix densities, but the preponderant species were the dark cells. Mori and Leblond (1970) and Imamoto et al. (1978) investigated the ultrastructure of OLGs in the corpus callosum of young rats. They too noticed variability in size and electron density among cells that had all the trademarks of OLGs. On the basis of these observations, Mori and Leblond defined three prototypes: light, medium, and dark OLGs distinguished by their decreasing size and increasing cytoplasmic density in the sequence of light to medium to dark (Figure 2-6). The implication of this work is that these different forms represent maturational stages in the development of OLGs and hence functional specialization. It is conceivable that the light OLG, which is observed myelinating axons, is the myelin-making cell, whereas the dark OLG is the myelin-maintaining cell. Such an operational definition can be supported by biochemical evidence (Szuchet et al., 1983, 1986). The notion that OLG fine structure varies as the cell changes its function from making to maintaining myelin is an attractive one. A strong case for this hypothesis was advanced by Parnavelas et al. (1983), who performed a detailed developmental study of the rat. They observed that for the deep cortex and subcortical white matter OLGs underwent a sequential transformation from light to dark forms so that in the mature animal 90% of the cells are of the dark variety. However, these results cannot be generalized to all areas of the central nervous system because in spinal cord white matter medium-type OLGs are encountered even in the adult animal.

The difference in cytoplasmic density in OLGs, which is normally attributed to the richness in ribosomes, cannot be due to the latter because ribosomes are more abundant in light than dark OLGs. The opposite appears to be the case for cisterna of the endoplasmic reticulum and Golgi saccules. The existence of these OLG variants has been amply confirmed (Peters et al., 1991) not only in mammals but in amphibians (Stensaas and Stensaas, 1968b),

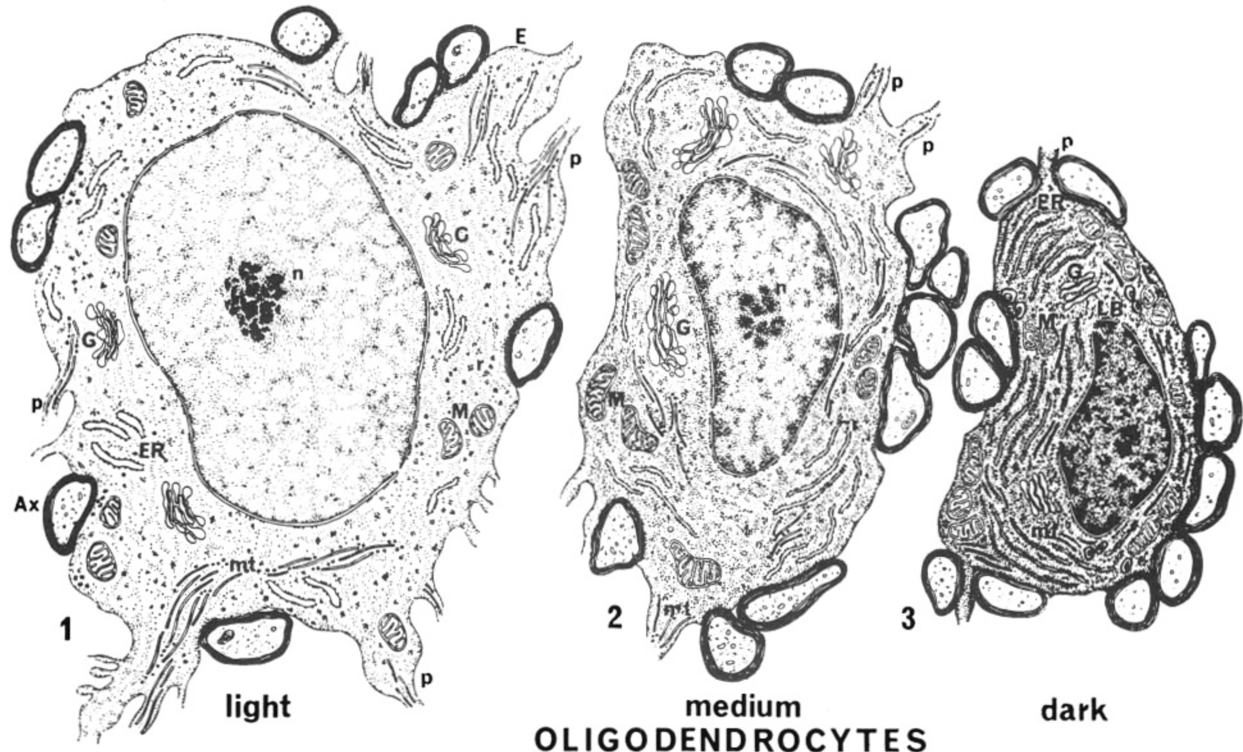


FIG. 2-6. Different types of oligodendrocytes found in the corpus callosum of young rat. 1, light; 2, medium; 3, dark. *n*, nucleolus; *mt*, microtubules; *r*, ribosomes; *G*, Golgi apparatus; *ER*, endoplasmic reticulum; *M*, mitochondria; *p*, process; *E*, extensions of cytoplasm; *Ax*, axons; *LB*, lamellar bodies. [From Mori and Leblond (1970), with permission.]

teleosts (Diaz-Regueira et al., 1992), and reptiles (Monzon-Mayor et al., 1990) as well.

Three-Dimensional Reconstruction. Classical electron microscopy provides a two-dimensional view of a cell, unless sequential electron micrographs are taken and reconstructed. Stensaas and Stensaas (1968b) undertook such a task and obtained OLG morphologies (Figure 2-7) that reproduce the images of type I and II OLGs as viewed by Golgi-Rio Hortega staining. Recently, Remahl and Hildebrand (1990) performed a detailed analysis of the ultrastructure and three-dimensional conformation of OLGs in developing feline spinal cord and corpus callosum. These areas correspond, respectively, to areas that are myelinated early and late during ontogenesis. Their results—discussed further in the section *The In Vitro Model* . . . —challenge several currently held views.

Two types of cell morphologies were seen in the spinal cord by Remahl and Hildebrand (1990). One is that of a cell connected to the myelin of a single axon (Figure 2-8A). This cell has all the characteristics of a type IV OLG (compare Figure 2-8B with Figure 2-1D). Formally, the relationship of this cell to its myelin sheath (Figure 2-8B) resembles the Schwann cell-myelin association. To the best of my knowledge, this is the first direct electron micrographic confirmation of the existence of such cells. Stensaas and Stensaas (1968a) detected structures—tubular reticulates—with a similar appearance, but they could not identify somata related to them. The other form has processes in continuity with uncompacted cytoplasmic ensheathements (see Figure 2 of Remahl and Hildebrand, 1990). This observation, if confirmed, would call into question the concept that OLGs do not ensheathe axons (R. Bunge et al., 1978; see also the section *The In Vitro Model* . . . later in this chapter.)

In the corpus callosum, the only variants detected fit the most common view of OLGs, that is, a cell associated with many myelin sheaths (Figure 2-9). Such cells would belong to type I or II. Remahl and Hildebrand (1990) made no distinction between them.

OLIGODENDROCYTE SUBTYPES: STRUCTURAL ODDITIES OR FUNCTIONAL ENTITIES?

The notion that OLGs are responsible for making and maintaining myelin rests on solid ground (Peters

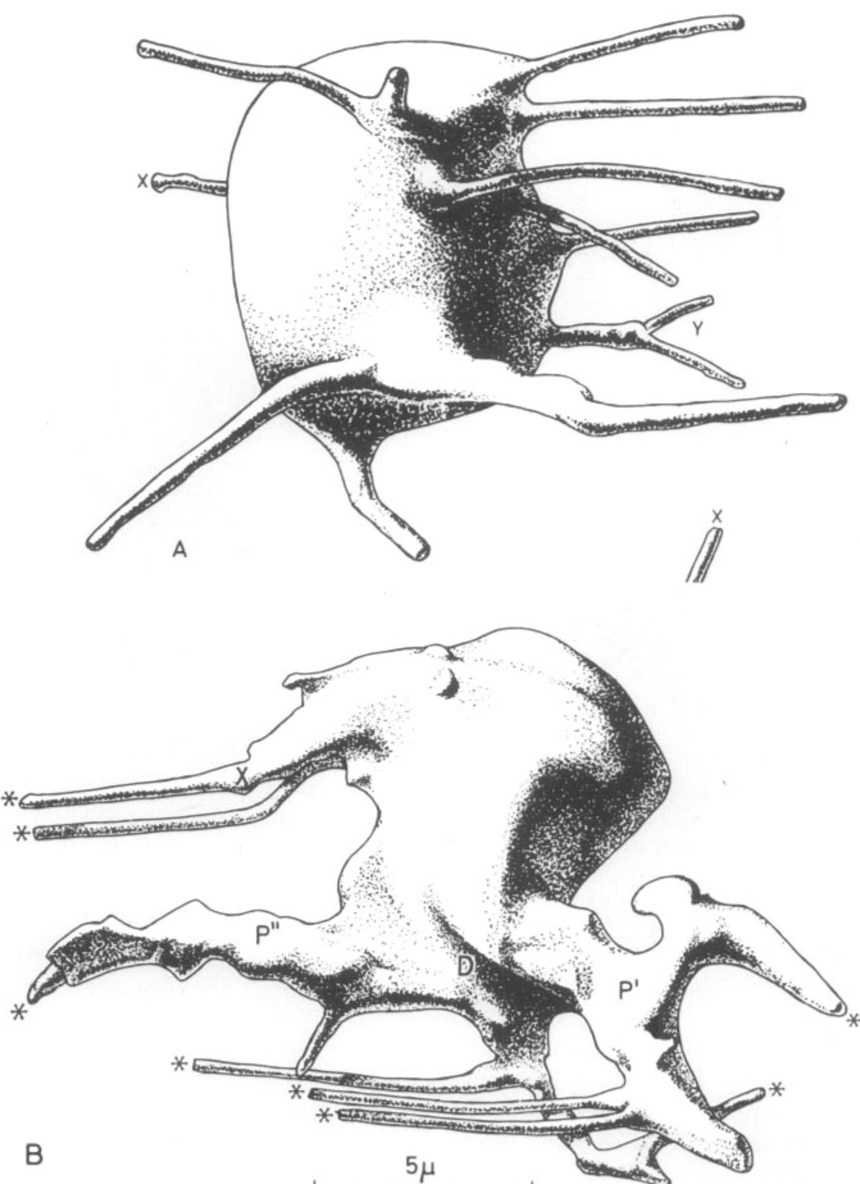


FIG. 2-7 Views of oligodendrocytes reconstructed from serial electron micrographs. (A) and (B) show different cells. These cells resemble Rio Hortega type I or II oligodendrocytes. Processes marked with asterisks are incomplete because they extend beyond the limits of the serial photomicrographs. [From Stensaas and Stensaas (1968), with permission.]

et al., 1991). The evidence is also strong that OLGs constitute a heterogeneous population of cells. This heterogeneity is expressed in more than one way. It is revealed in OLG-axon interactions where associations of 1:1 (type IV OLGs; Figure 2-8) to 1:N (types I or II; Figure 2-9) are formed. This in itself bespeaks functional diversity among myelinating OLGs. First, it was shown by Hartman et al. (1982) that large axons stain more intensely for myelin basic protein than proteolipid protein, whereas the reverse is the case for small axons. Second, compositional differences between myelin isolated from spinal cord and that obtained from the brain have also been described (Norton and Cammer, 1984). Since the spinal cord is populated predominantly by type IV OLGs (Remahl and Hildebrand, 1990), these OLGs may have a different metabolism from those found in the brain. Moreover, myelin changes in composition as it matures, which signifies that OLGs must adjust their metabolism to that effect (Szuchet et al., 1983; Norton and Cammer, 1984). On the surface, these dissimilarities may not seem profound, but their presence foreshadows differences in gene control and expression.

The cell that approaches an axon to commence myelination has an undifferentiated ultrastructure (Remahl and Hildebrand, 1990). The issue is whether the fate of such a cell to become a given OLG subtype, for example, type IV, is predetermined by genetic programming and/or is influenced by its microenvironment. For instance, is the cross-talk between neurons and OLGs in the spinal cord different

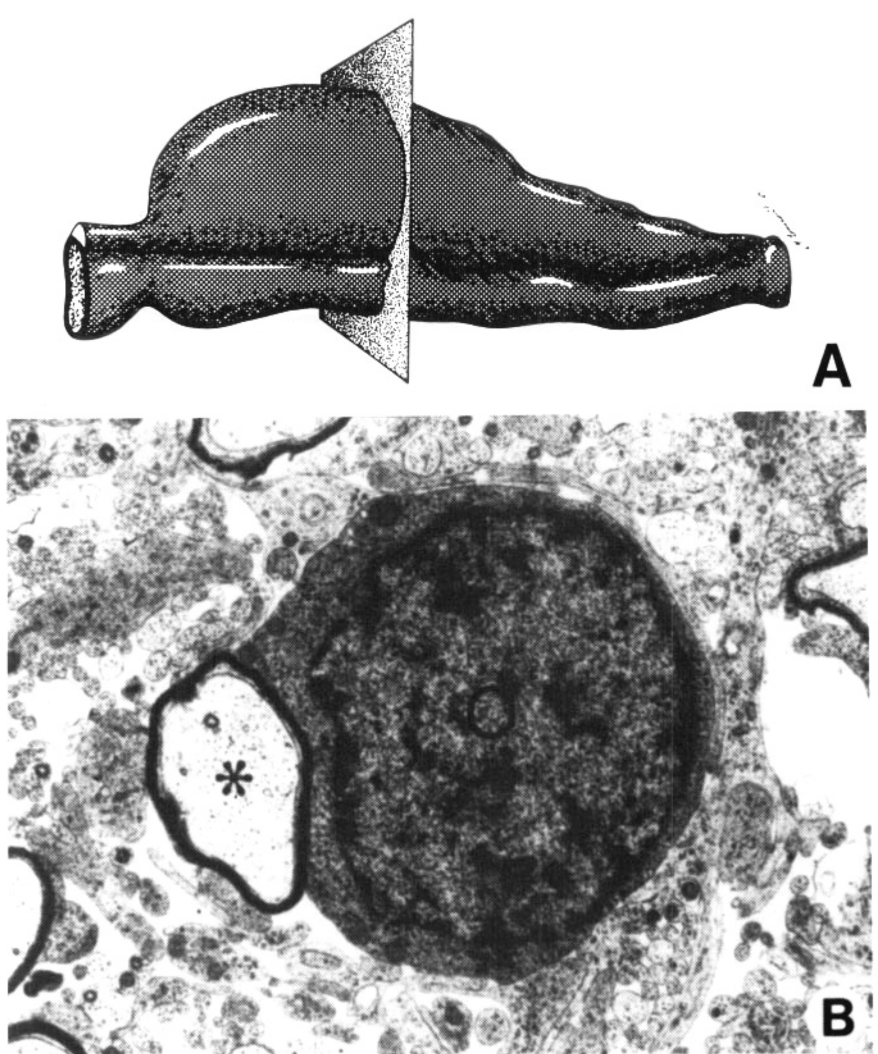


FIG. 2-8. Oligodendrocyte from feline spinal cord of 47-day-old fetus. (A) An M-type glial unit, which includes one axon. Note the close relation between the perikaryon and the myelin sheath that extends along the whole cell body. (B) This electron micrograph corresponds to the transverse plane marked on Figure 8A. O indicates the nucleus of the oligodendrocyte linked to one axon with an M-sheath (*). [From Remahl and Hildebrand (1990), with permission.]

from that in the brain? It is a common finding that the thickness of a myelin sheath is related to the girth of the axon it invests; the thicker the axon, the thicker the myelin. Indeed, the same OLG may produce myelin of different thickness according to the size of the axons it wraps (Friedrich and Mugnaini, 1983; but see Remahl and Hildebrand, 1990). It has been suggested that the total amount of myelin synthesized by an OLG is relatively constant (Blakemore, 1981). These findings have been used to argue that it is the neuron that regulates myelination (Blakemore, 1981; Waxman and Sims, 1984; Waxman, 1987). These concepts have been challenged by recent observations, indicating that the amount of myelin an OLG assembles may be primarily determined by its type and only indirectly by the size of the axon (Remahl and Hildebrand, 1990).

There are OLGs that are not involved in the myelination process under normal circumstances, for example, perineuronal OLGs. One may wonder what it is they do. It has been shown that such OLGs have the potential to myelinate and seem to do so under pathological circumstances (Ludwin, 1984), but this is not their normal function. Moreover, in species where central nervous system regeneration takes place (e.g., fish), Diaz-Regueira et al. (1992) found no perineuronal OLGs in the medulla. The suggestion has been made that satellite OLGs may specialize in tending a specific type of neuron, for example, Purkinje cells (Monteiro, 1983). Should this be the case, it would imply a greater OLG diversity than what is currently envisioned. In a similar vein, the complexity of processes displayed by "perivascular" OLGs (see Figure 18 in Rio Hortega, 1928) makes it difficult to conceive that they have no specific function, even though they may not actually abut a capillary. In the adult mammal, there is strong evidence for the existence of a thin sheet of astrocytic membrane interposed between the OLG and the blood vessel (Mugnaini and Walberg, 1964; Nagash-

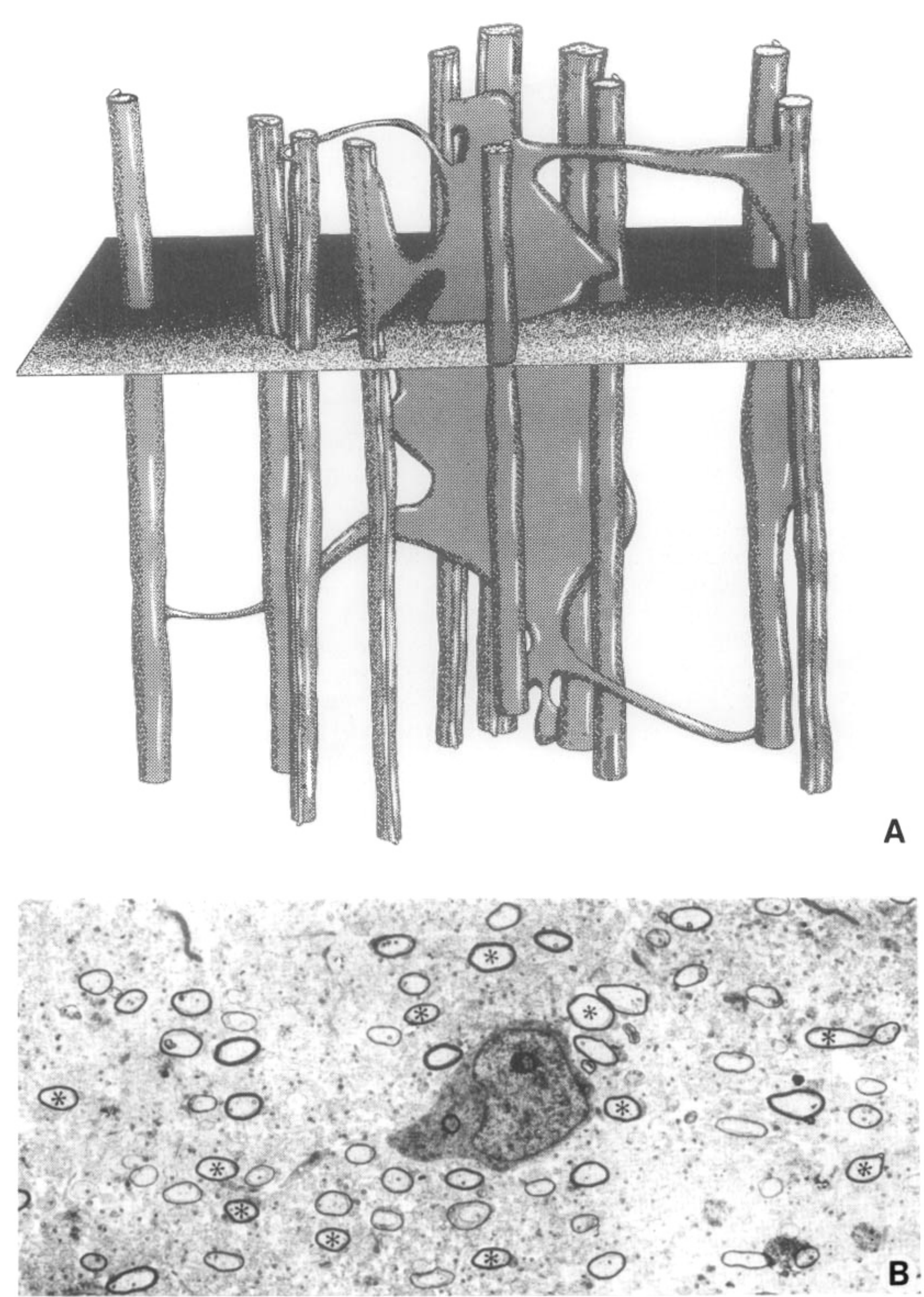


FIG. 2-9. Oligodendrocyte from feline corpus callosum, 21 days postnatal. (A) An M-type glial unit including 11 axons. (B) This picture corresponds to the transverse plane marked on Figure 9A. The 11 axons are indicated by asterisks and the oligodendrioglial cell body (O) and nucleus are clearly seen. [From Remahl and Hildebrand (1990), with permission.]

ima, 1979). This appears to be the case for other species as well (Long et al., 1968). Unfortunately, we know very little about what the function of these so-called "perivascular" OLGs might be.

It would be outside the scope of this chapter to discuss the function of OLGs in a general sense (see Kuffler et al., 1984; Peters et al., 1991). My intent here has been to point out that morphology may be only one factor that differentiates the various OLG subtypes; function may be the other. Functional specialization of OLG subtypes could perhaps account for what now stand out as discrepancies. For example, using freeze-fracture electron microscopy, Massa et al. (1984) found tight junctional complexes between OLGs; no gap junctions were detected. These results are in line with findings *in situ* (Massa and Mugnaini, 1982) but are at variance with the observations of Blankenfeld et al. (1993), who described electrical and dye coupling of OLGs as they mature. Butt and Ransom (1989) obtained similar results for OLGs from the optic nerve. The electrophysiology of OLGs is also marred by some controversy (e.g., see Soliven et al., 1988, 1989; Kettenmann et al., 1991). There may be a single explanation for this, and it is that different OLG subtypes were being examined. Thus it may prove rewarding to characterize OLGs from specific areas of the central nervous system. Suggestions have been advanced that astrocytes from different central nervous system regions may have distinctive properties (Ransom, 1991).

THE *IN VITRO* MODEL: MORPHOLOGY OF IMMATURE VERSUS MATURE OLIGODENDROCYTES AND FUNCTIONAL IMPLICATIONS

OLGs can be isolated from neonates as typified by the procedure of McCarthy and de Vellis (1980) or they can be isolated from young but myelinated animals as exemplified by the method of Szuchet et al. (1980a) (see also Shahar et al., 1988). OLGs can be maintained as pure cultures (Szuchet et al., 1980) or as a mixed population of cells (review by Pfeiffer, 1984). These cultures afford a unique opportunity to investigate the effect of the microenvironment and/or other cell types on OLG morphology and ultrastructure.

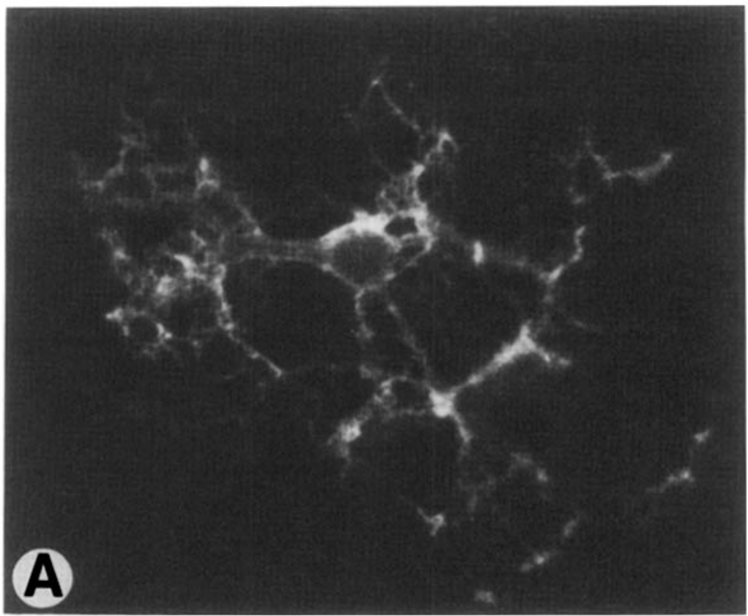
The Immature Oligodendrocyte

Perusal of the literature reveals that OLGs isolated from rat or mouse neonates have a distinct morphology (Figure 2-10A) that appears to be independent of the details of methodology or the medium utilized. The cell soma is either round or polygonal and has three to five stout processes emerging directly from the body, each of which produces an expansive network of fine processes and membranes. Such cells stain positively for galactocerebroside—an OLG-specific marker—and express myelin components. The immature cells exhibit little or no similarity to the OLGs illustrated in Figure 2-1; however, the cell in Figure 2-10A shows a striking resemblance to the mossy OLGs (compare Figure 2-10A with Figure 2-10B) described by Ramón-Moliner (1958). The mossy cells can be found in white and gray matter with essentially no morphological distinction between them. The mossy cell has not been detected in mature animals. Ramón-Moliner speculates that it represents an immature form of OLG capable of differentiating into a myelin-forming cell, as evidenced by the acquisition of parallel processes (see Figure 4 in Ramón-Moliner, 1958). Immature OLGs, when kept in long-term cultures, assemble myelinlike membranes (e.g., Bradel and Prince, 1983), which supports the view held by Ramón-Moliner (1958).

Kuhlmann-Krieg et al. (1988) attempted to correlate the morphology and ultrastructure of cells isolated from 10-day-old mice with surface antigens that define successive stages of OLG differentiation. They concluded that the *in vitro* morphology may be more a reflection of metabolic states than differentiation states. The recent findings of Remahl and Hildebrand (1990) indicate that the biochemical differentiation of OLGs, as assessed by the expression of myelin-specific components, precedes the morphological differentiation. Thus the cell that begins myelination appears as morphologically undifferentiated. Only after some myelin has been deposited does the cell acquire the ultrastructure that is its trademark.

The Mature Oligodendrocyte

OLGs (isolated from postmyelination brains) adhered to a suitable substratum and kept in culture (Szuchet et al., 1983; Yim et al., 1986) essentially free of other cell types acquire morphologies that resemble those seen *in situ* (Figure 2-11). Illustrated in Figure 2-11 are morphologies that approximate OLGs of types I, II, and III of Rio Hortega (1928). This phase-contrast micrograph was taken from a 90-day-old culture. Such cultures can be thought of as "myelinating" because they contain a large number of multilamellar membranous structures (Szuchet et al., 1986). The most striking feature of Figure 2-11 is the presence of long parallel processes not unlike the processes OLGs extend along nerve fibers (compare Figure 2-11 with Figure 2-2). The connotation



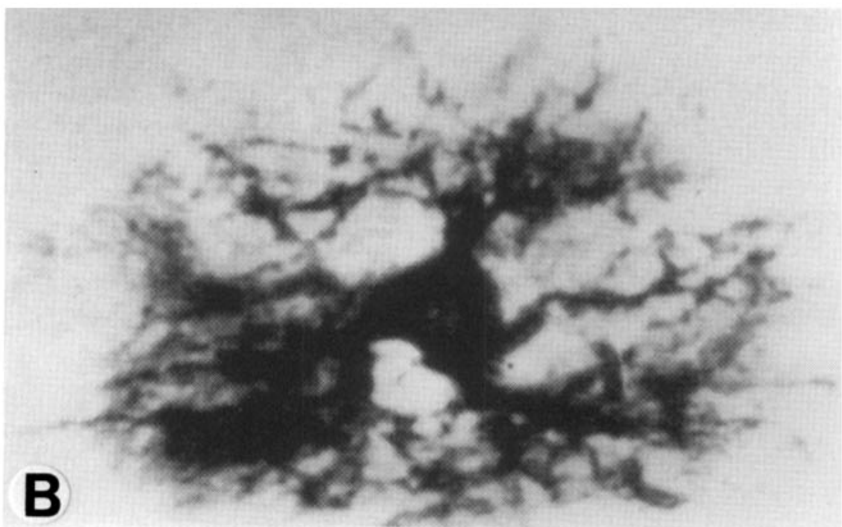


FIG. 2-10. Immature oligodendrocytes. (A) Oligodendrocyte in a culture of dissociated 19- to 21-day fetal rat brain, after 22 days in culture, stained by immunofluorescence with antigalactocerebroside. [From Pfeiffer (1984), with permission.] (B) Mossy type of oligodendrocyte. Golgi-Rio Hortega stain. [From Ramón-Moliner (1958), with permission.]

here is that this seems to constitute an intrinsic property of OLGs poised for myelination and does not require the presence of an axon. However, most of these OLGs should have been in contact with an axon prior to their isolation, which leads to two alternative possibilities: (1) OLGs need a signal from axons to differentiate morphologically, but they need it only once; and (2) the signal is always required, but substratum can function as a surrogate. The second possibility appears to be the most likely (Szuchet et al., 1983; Yim et al., 1986).

The different OLG morphologies developed *in vitro* can be appreciated better when the cells are outlined immunocytochemically (Figure 2-12) than when live cells are examined. In Figure 2-12A, a type I OLG is shown with processes radiating from the cell and branching out to cover an extensive area. One may speculate that this OLG might have occupied a perineuronal or "perivascular" position. Figure 2-12B depicts a number of closely apposed OLGs with all the morphological characteristics of type II. These cells could clearly claim a place among the interfascicular OLGs. An example of a type III OLG is portrayed in Figure 2-12C. Three sturdy processes emanate from the cell body, two of which bend in a direction perpendicular to the initial course to run parallel to one another. Two monopolar cells are illustrated in Figure 2-12D; they should correspond to type IV OLGs. These cells have been stained with an antimyelin basic protein Ab, which attests to their identity as OLGs. As is the case *in situ*, the predominant classes are types I, II, and III (possibly in this order). Type IV is rarely found. This agrees with the observation of Remahl

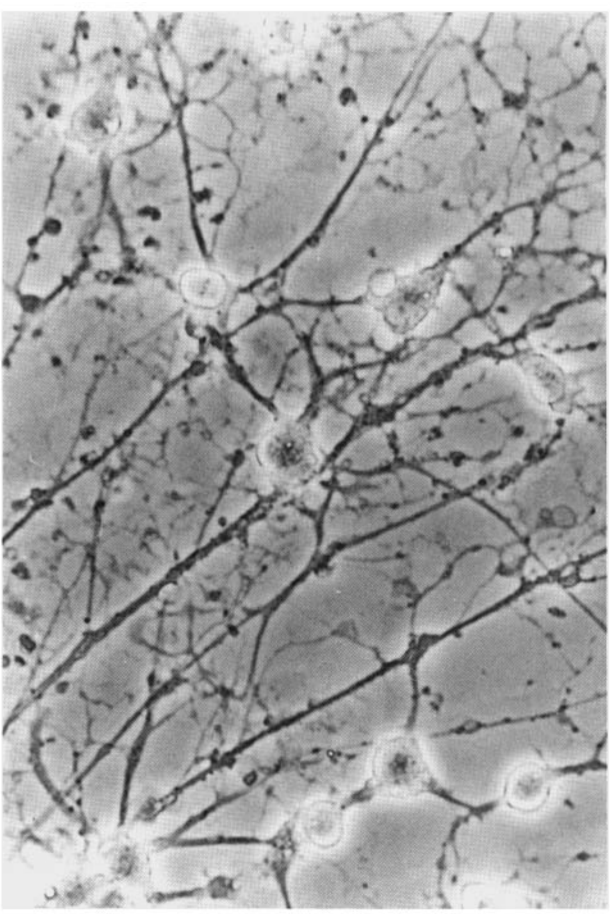


FIG. 2-11. Phase-contrasted micrograph of live oligodendrocytes; 90-day-old culture. Of note are the long, parallel processes and the varied morphology of the cells. [S. Szuchet, unpublished data.]

and Hildebrand (1990), who only detected type IV OLGs in the spinal cord. The cultures described originate from brain white matter. It is interesting that all these morphologies are observed. The fact that this type of heterogeneity has not been described in cultures of neonates could be taken to indicate that neurons may have a say in the manner in which OLGs develop, and this becomes imprinted in the cells. So even after they have been separated from the inducer they reenact whatever morphology they had *in situ*. This line of reasoning leads one to speculate that each of these morphologies might be endowed with distinct functional characteristics.

The ultrastructure of cultured OLGs has been investigated by a number of laboratories (e.g., Wollmann et al., 1981; Gonatas et al., 1982; Massa et al., 1984; Kuhlmann-Krieg et al., 1988). The consensus is that the ultrastructure of the cultured cells parallels closely the characteristics of the *in situ* cells. Wollmann et al. (1981) examined OLGs isolated from 3- to 6-month-old lamb brains. They observed that the freshly isolated cells were more electron-lucent than cells from the same animal examined in tissue sections. The authors attributed this to a dedifferentiation of the isolated cells. However, after adhesion to a substratum and extension of processes, OLGs acquired characteristics similar to those seen *in situ*. Variation in electron density as described by Mori and Leblond (1970) was seen with a predominance of the medium OLG.

The studies of Wollmann et al. (1981) were extended by Massa et al. (1984) to encompass three experimental stages: (1) freshly isolated OLGs; (2) OLGs maintained in a nonadhered state; and (3) adhered OLGs. Each of these stages could be equated operationally to myelin-maintaining OLGs, dedifferentiated OLGs, and myelin-making OLGs (Szuchet et al., 1983; Szuchet and Yim, 1984; Yim et al., 1986). These studies placed particular emphasis on cell-cell interactions as viewed by freeze-fracture electron microscopy. Only the remarkable features will be presented.

In thin section, the freshly isolated OLGs appeared to be moderately electron-dense; the cytoplasm contained the usual complement of intracellular organelles. Of salience were intracellular vacuoles with electron-dense membranes and blebs on the plasmalemma that mark sites of adhesion to neighboring cells. Intramembrane particles revealed by freeze-fracture electron microscopy resembled qualitatively and quantitatively those found in OLGs *in situ* (Massa and Mugnaini, 1982). Where two OLGs came in close apposition, there was an enrichment of intramembrane particles. Gap junction and tight junction particles occurred frequently, with the former predominating. Fragments of blebs that have the orthogonal arrays characteristic of astrocytic membranes were often associated with the gap junctions. This finding was interpreted as showing that *in situ* OLGs were coupled to astrocytes (Massa and Mugnaini, 1982).

The nonadhered OLGs showed round cell somata without processes. The cytoplasm contained numerous microtubules. Overall the cells resembled OLGs described in the brains of young mammals (Mori and Leblond, 1970). Nonadhered OLGs were apposed to one another, but no junctional particles were detected.

Upon adhesion to a substratum, OLGs differentiate morphologically by extending numerous cylindrical and flattened processes. OLGs were examined after 1 to 5 weeks in culture. In thin section, outstanding features were a prominent Golgi apparatus, an abundance of microtubules, and electron-dense bodies. Coated pits were frequently observed on the

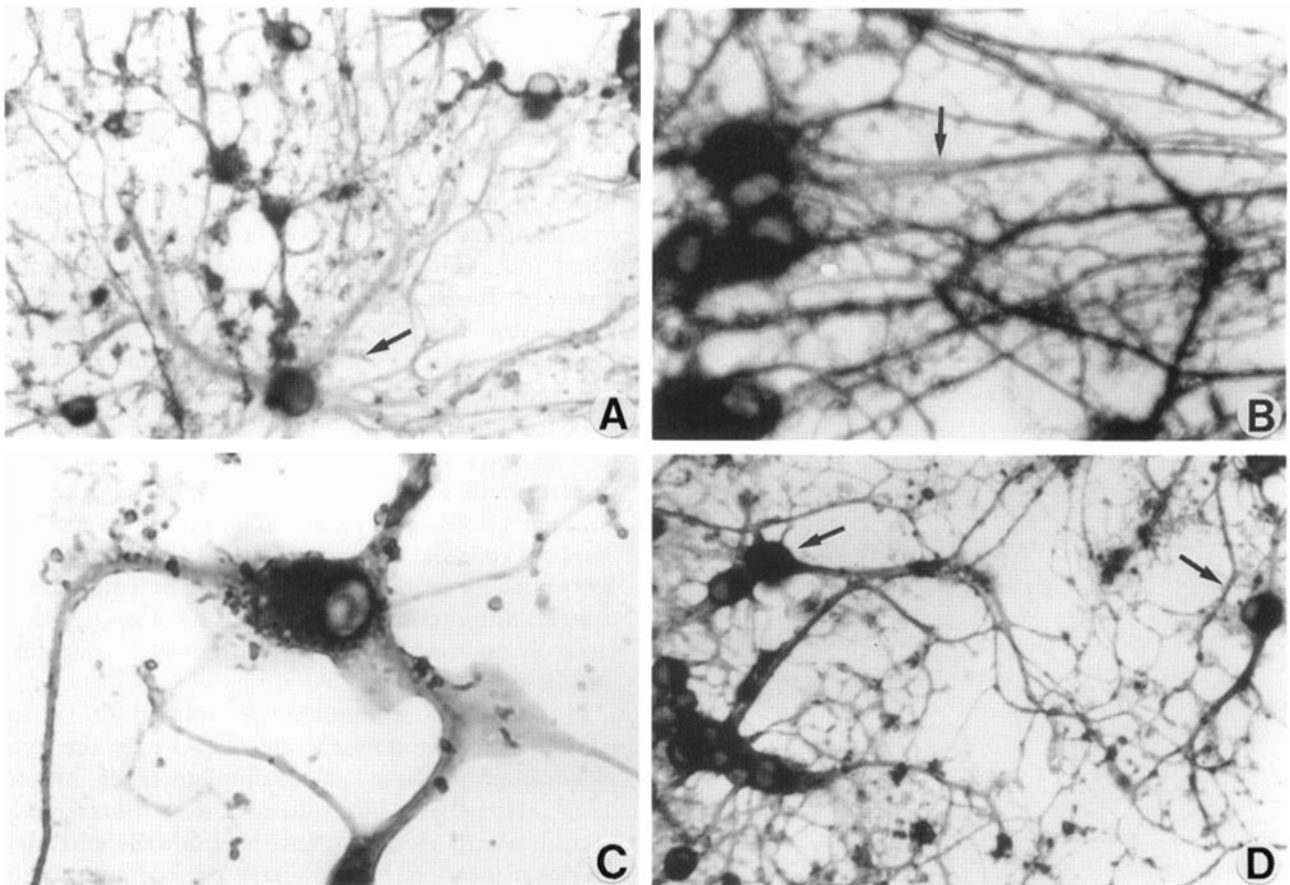


FIG. 2-12. Micrographs of cultured oligodendrocytes stained with antimyelin basic protein. (**A**) Oligodendrocyte with 4 to 6 hefty processes emerging from the soma (*arrow*) and branching out. This cell resembles type I oligodendrocyte. (**B**) Group of closely apposed oligodendrocytes with parallel processes (*arrow*), resembling type II oligodendrocyte. (**C**) Oligodendrocyte with morphological features of type III. This oligodendrocyte has three sturdy processes emanating from the cell body. (**D**) Type IV oligodendrocyte. *Arrows* point to cells with a type IV morphology.

plasma membrane that apposed the substratum and other OLGs. Freeze-fracture electron microscopy revealed globular and elongated particles distributed on the P and E faces. Linear arrays that resembled the peculiar tight (occluding) junctions of myelin (Schnapp and Mugnaini, 1978) were often seen. Their numbers increased with time in culture. In contrast, no gap junctions were found. These observations suggest that OLGs require an input from astrocytes for the synthesis of gap junctions.

EXTRINSIC AND INTRINSIC FACTORS THAT MODULATE OR DETERMINE OLIGODENDROCYTE MORPHOLOGY

Trophic Factors

Our knowledge of the cues that guide OLGs in their path toward differentiation is meager at best. That a neuronal input is required stems from the now classical experiments of Fulcrand and Privat (1977), who showed that, in early stages of OLG differentiation, they seem to require an interaction with neurons. However, the nature of this interaction has never been defined. *In vitro,* hormones (de Vellis, 1990) and growth factors (Raff and Lillien, 1988; Hart et al., 1989; McKinnon et al., 1990; McMorris et al., 1990) influence the differentiation of OLGs; some of them appear to be operative *in situ* as well. Paradoxically, because OLGs maintain their phenotype in the absence of other central nervous system cell types and behave permissively with respect to substrata, their requirements for biochemical and morphological differentiation have been difficult to establish. This is to be contrasted with Schwann cells, whose trophic wants are proving to be easily decipherable (R. Bunge et al., 1986) because they lose their phenotype in the absence of (1) an input from neurons, (2) an extracellular matrix, and (3) certain nutrients.

The *in vitro* changes in OLG morphology induced by growth factors have been documented by several laboratories (e.g., Kachar et al., 1986; Sontheimer et al., 1989). For example, platelet-derived growth factor (PDGF) can drive the cells reversibly from a stellate to a bipolar morphology (McKinnon et al., 1993). These authors also found that cell shape was associated with the expression of specific markers. A similar observation was made by Warrington and Pfeiffer (1992), using tissue blocks. This, however, has not been the experience of Kuhlmann-Krieg et al. (1988).

Effect of Substratum on Morphological Differentiation

Cell-substratum adhesion plays a crucial part in the cascade of events that control growth or turn on and consummate a differentiation program. For those cellular systems that amass an extracellular matrix, the latter fulfills a complex set of functions that include (1) facilitating access to growth factors, (2) controlling the activity of proteases-protease inhibitors involved in tissue modeling-remodeling, and (3) providing a host of adhesion molecules and other ligands (review in Hay, 1991). The importance of an extracellular matrix for the differentiation of Schwann cells and for myelination in the peripheral nervous system has been clearly established (M. Bunge and Bunge, 1986; R. Bunge et al., 1989). Extracellular components such as proteoglycans and adhesion molecules have been shown to be present in the central nervous system (Herndon and Lander, 1990; Rauch et al., 1991, 1992; Grumet et al., 1993). For example, polyclonal and monoclonal Abs against astrochondrin—a newly characterized chondroitin sulfate from brain (Streit et al., 1993)—have been shown to inhibit formation of processes of mature glial fibrillary acidic protein-positive astrocytes on laminin or type IV collagen. No direct information is available as to what role, if any, extracellular matrix components play in the development of OLGs and in central nervous system myelination. Circumstantial evidence suggests that they may play a major part. Yim et al. (1993) have demonstrated that the adhesion of OLGs to substratum induces the synthesis of both peripheral, for example, displaceable by heparin, and integral heparan sulfate proteoglycans. The peripheral heparan sulfate proteoglycans are incorporated asymmetrically into the plasma membrane, leading to its polarization; their removal prevents cell adhesion. These authors suggest that heparan sulfate proteoglycans are key molecules in the morphological differentiation of OLGs.

In vitro, the signal for the biochemical and morphological differentiation of postmyelination OLGs arises from adherence to an adequate substratum (Szuchet et al., 1983; Szuchet and Yim, 1984; Yim et al., 1986). Isolated OLGs kept in a nonadhered state, for example, plated on tissue culture plates, form aggregates (see the section *The In Vitro Model* . . .) but fail to extend processes or differentiate biochemically (Vartanian et al., 1986; Yim et al., 1986). OLGs behave selectively toward substrata. They adhere poorly on collagen, laminin, and fibronectin, characteristics that set them apart from Schwann cells and neurons. Native vitronectin is not effective either, but exposure of the cryptic heparin-binding domain of vitronectin by urea denaturation converts it into an acceptable substratum (Schirmer et al., 1993). Ovadia et al. (1984) reported that when OLGs adhered to an extracellular matrix generated by bovine corneal endothelial cells, they underwent proliferation and differentiation. Laminin and other extracellular matrix components were believed to be responsible for this effect. However, OLGs were loosely attached and tended to form clusters when plated on purified laminin. These authors also found collagen to be ineffective. Cardwell and Rome (1988a, 1988b) found an extracellular matrix derived from glial cells lysed with H_2O (astroglial matrix) to be an effective substratum for rat OLGs. Ovine OLGs express their myelinogenic repertoire when plated on a polylysine-coated surface in the presence of 20% horse serum (Szuchet and Yim, 1984). Schirmer et al. (1994) have purified a novel, heparin-binding glycoprotein from horse serum, which they have termed GRASP—glycine-rich adhesion serum protein. When GRASP is coated on plates precoated with polylysine, it promotes the differentiation of OLGs in DMEM alone, that is, in the absence of serum. However, when growth factors, hormones, and metals are added to the DMEM, OLGs prosper as well and even better than with 20% horse serum. Schirmer et al. (1994) speculate that an integrinlike molecule and heparan sulfate proteoglycans are involved in the GRASP mode of action.

Influence of Cell-Cell Interaction on Oligodendrocyte Morphology

No systematic studies have been performed that define the influence astrocytes and/or neurons might have on OLG morphological development.

Neuronal Input: How Is It Manifested? In the peripheral nervous system neuronal input is expressed by direct physical contact between the Schwann cell

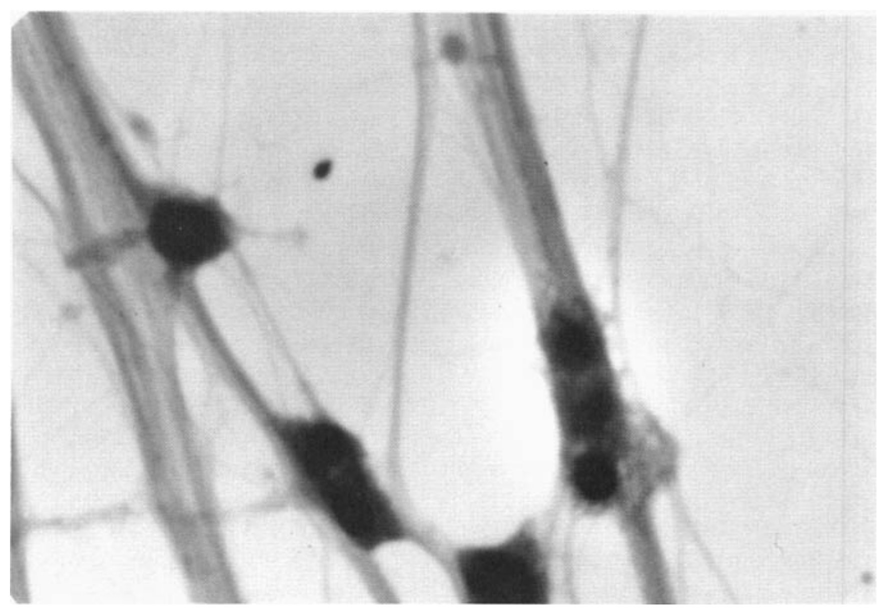


FIG. 2-13. Adherence of oligodendrocytes on dorsal root ganglion neurites. Isolated ovine oligodendrocytes were added to rat dorsal root ganglion cultures. Oligodendrocytes adhered preferentially on the neurites and formed rows of closely apposed cells, not unlike interfascicular oligodendrocytes. Noteworthy is the fact that cells have extended processes along the neurites. Oligodendrocytes that stayed on the substratum (collagen) formed clumps. No processes emanated from the latter cells. Stained with toluidine blue. (P. Wood and S. Szuchet, unpublished observations.]

and the neuron. I have already alluded to the work of Fulcrand and Privat, wherein it was noted that OLGs need a neuronal input. These experiments do not indicate whether physical contact between the two cell types is needed or whether the influence is transmitted via a secreted factor. Also, OLGs appear to do well *in vitro* in the absence of neurons, even when isolated as precursor cells, which lends an element of uncertainty to the notion of neuronal influence. That OLGs choose to adhere to neurons over another substratum can be inferred from the results of adding isolated OLGs to neuronal cultures. OLGs adhere preferentially to the neurite and develop morphologically by extending processes along the nerve fibers (Figure 2-13). In contrast, OLGs that remained on the substratum (collagen) formed clumps without extending processes. These results point strongly to a surface interaction between the two cell types.

Astrocytic Influence. *In situ,* OLGs and astrocytes are coupled by gap junctions (Massa and Mugnaini, 1982). As mentioned above, freshly isolated OLGs carry the remnants of such junctions. However, once OLGs adhere and develop in culture in the absence of astrocytes, they no longer seem to express the component necessary for the formation of gap-junctional complexes (Massa et al., 1984). These observations can be interpreted as indicating that OLGs need an input from astrocytes in order to assemble gap junctions. That OLGs appear to be able to accomplish their myelination repertoire without gap junctions suggests that the latter subserve a function that may not be directly related to the formation of myelin. Alternatively, the *in vitro* environment compensates for the lack of junctions.

Oligodendrocyte-Oligodendrocyte Interaction. This topic is brought up only for the sake of completeness. We know little to nothing on this issue. *In situ,* OLGs align in rows of closely apposed cells; tight junctions have been found between these cells (Massa and Mugnaini, 1982). Again, remnants of these particles are detected in the freshly isolated OLGs (Massa et al., 1984). Significantly, *in vitro* tight junctions are also formed between the cells organized in rows, not unlike the situation *in situ.* The manner in which these junctions contribute to the structural organization and/or function of OLGs remains an open question.

Cultured OLGs secrete constitutively to the medium six sulfated glycoproteins and chondroitin sulfate proteoglycans that are temporally modulated. Their functions remain unknown. It is tempting to speculate that some of them might have an autocrine/paracrine function (Szuchet and Yim, 1990).

CONCLUSIONS AND PERSPECTIVES

In this chapter I have described OLG morphology and ultrastructure with particular emphasis on (1) the morphological polymorphism of OLGs, and (2) establishing a link between polymorphism and function. The data presented set the presence of polymorphism on firm ground. It is manifested *in situ* as well as *in vitro;* it is detected by a variety of techniques. Thus neuroscientists will have to come to

grips with the notion that OLGs are not a homogeneous population of cells; rather, they are organized in subtypes with selected locales for each of them. It required roughly sixty years for the findings of Río-Hortega to be revindicated. The elegant work of Remahl and Hildebrand established the first link between morphology and functional specialization. Clearly this only marks the beginnings. Efforts should now be directed toward isolating OLGs from specific regions and characterizing their biology.

It is conceivable that the biology of a type IV OLG bears more resemblance to that of a Schwann cell than to a type II OLG. However, type IV OLGs are not Schwann cells: they lack a basal lamina, which is essential for the proper function of Schwann cells. Since the assembly of a basal lamina by the Schwann cell is under the control of the neuron, it implies that the lines of communication between type IV OLGs and neurons are different from those utilized by the Schwann cell-neuron connection. Superimposed on the morphological heterogeneity is an ultrastructural heterogeneity. This may be the result of changes in the metabolism associated with making and maintaining myelin. Another area that needs to be addressed is that of the satellite OLGs, that is, perineuronal and "perivascular." Are they keepers of the microenvironment? Such studies, however, may have to await new technological advances before meaningful attempts can be made.

The factors that influence OLG growth and differentiation remain largely undefined. In particular, we know very little of what the neuron does. When and how do these two cell types talk to each other? Does the neuron have a say about whether an OLG will become type IV or type II, or is this information imprinted in the genetic makeup of the cell? What part does the microenvironment play? If the assertions of Remahl and Hildebrand are correct, then type IV OLGs appear before type II. This would indicate that the fate of an OLG subtype may be established ontogenically. Tissue culture, if interpreted with due reservations, could well serve as the vehicle for addressing many of the outstanding questions.

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3 Basic biology of the Schwann cell

RICHARD P. BUNGE AND CRISTINA FERNANDEZ-VALLE

Essentially everywhere in the peripheral nervous system glial cells are interposed between nerve cell bodies and fibers and the surrounding nonneural tissues. Within sensory and autonomic ganglia, these cells assume perineuronal positions as satellite cells. Within the ganglia of the enteric plexuses of the gut, glial cells encase the neuronal aggregates in a manner similar to the disposition of astrocytes of the central nervous system. Within the peripheral nerve trunk, they ensheathe all nerve fibers, whether myelinated or unmyelinated. These glial cells of the peripheral nerve trunks, the cells of Schwann, are the subject of this chapter.

Schwann cells provide housing for axons within peripheral nerves. Part of this housing function is the formation of an interface between the neural tissue and surrounding tissues. Thus, around each axon-Schwann cell unit there is invariably a basal lamina, which delineates the border with adjacent connective tissue components. This arrangement allows a peripheral nerve to contain considerable amounts of connective tissue to provide the requisite physical strength and yet retain within it tubular channels for the delicate nerve fibers. In addition to this function, Schwann cells form myelin sheaths along the larger nerve fibers. The deposition of myelin in segments influences the location of sites allowing ionic current passage in the enclosed nerve fiber; this partitions the axolemma, concentrating certain ion channels at the intervening nodes of Ranvier. This positioning allows saltatory conduction of the nerve impulse, which increases the velocity of nerve conduction as much as 100 times. Beyond these basic functions, the Schwann cell is able to respond to nerve injury by helping to remove cellular debris, by facilitating the process of remyelination, and by promoting nerve fiber regeneration. For almost a century the presence of Schwann cells in the peripheral nerve trunk and their absence in the central nervous system has been considered a primary factor in the differences in regenerative capacity between these two tissues (see the translation of Rámon y Cajal's *Degeneration and Regeneration of the Nervous system,* first published in 1913–1914; Cajal, S. R., 1991).

SCHWANN CELLS IN EARLY DEVELOPMENT

Source of Schwann Cells

In the past decades it has been stated that Schwann cells derive from two structures transiently present during embryogenesis: the neural crest and the placodes of the head region (Harrison, 1924). Recent observations on chick/quail chimeras indicate that, with the exception of the olfactory placode, neural crest cells may be the source of all glial cells in the peripheral nervous system, including the Schwann cells of the peripheral nerve trunks, the satellite cells of sympathetic and parasympathetic ganglia, and the distinctive glial cells of the enteric ganglia. According to these observations, in most ganglia receiving placodal contributions only the nerve cells themselves are of placode origin, with all attending glia deriving from neural crest (for review, see LeDouarin et al., 1991; Ziller and LeDouarin, 1993). Schwann cells appear at a very early stage during the development of peripheral nerve and migrate outward along axonal fasciculi as the peripheral nerve trunks form (Tennyson, 1965; Webster and Favilla, 1984). Schwann cell populations for the ventral nerve roots are provided from neural crest cells migrating ventrally along the lateral aspect of the neural tube. There is no evidence that Schwann cells can be derived from other cell types subsequent to the embryonic period (Asbury, 1975). Schwann cells, however, maintain the ability to dedifferentiate following injury, and to proliferate, providing additional Schwann cells for the regenerative process (see below).

Early Stages of Development

In the earliest stages of peripheral nerve development, outgrowing bundles of axons are accompanied by small numbers of Schwann cells, which position themselves between the axons and the surrounding connective tissue, providing a partial intervening basal lamina (Ziskind-Conhaim, 1988). As will be discussed below, the number of Schwann cells

is influenced by contact with the axons and, as their numbers increase, they migrate interiorly into the axon bundles (Asbury, 1967; Webster, 1993). As the increasing numbers of Schwann cells isolate smaller and smaller bundles of axons, each axon-Schwann cell unit begins to acquire its own basal lamina, and thus the interior of the nerve begins to acquire its connective tissue components. Schwann cells related to the larger axons capable of inducing myelination position themselves along the axon length (R. Bunge et al., 1989). They then elongate and engulf a specific length of axonal domain prior to forming a membranous scroll around the axon that becomes the multilamellar myelin sheath. Smaller axons that are destined to remain unmyelinated induce their associated Schwann cells to differentiate into an ensheathing cell. These Schwann cells specialize in harboring one or several small-diameter axons within furrows on their surface. Eventually these axon-Schwann cell units are interspersed with collagen fibrils, fibroblasts, and ingrowing blood vessels. Groups of axon-Schwann cell units then become surrounded by a complex sleeve of flattened cells comprising the perineurium. This perineurial sheath forms a tissue/nerve barrier, which effectively excludes extraneural proteins from the internal regions of the nerve. It has recently been established that perineurial cells are recruited from the regional fibroblast population, and are not modified Schwann cells as had sometimes been suggested (M. Bunge et al., 1989a). The extracellular components within the perineurium compose the endoneurium. Both the Schwann cells and the fibroblasts contained within the endoneurium are known to contribute to the substantial amount of connective tissue deposited between each of the axon-Schwann cell units and which gives peripheral nerves their characteristic cord-like consistency (Obremski et al., 1993a, 1993b; reviewed in M. Bunge, 1993). Surprisingly, about one-third of the protein in a peripheral nerve is collagen. After enough Schwann cells have been generated to both myelinate the larger axons and ensheathe the smaller axons, the number of Schwann cells within the peripheral nerve trunk becomes stabilized (Asbury, 1967). As the nerve subsequently grows, new segments of myelin are not added to accommodate the growth; instead each of the myelin segments is extended in length so that myelin segments that may be 200 μm in length when first formed may subsequently reach 1000 μm in length.

Markers for Schwann Cell Subtypes

Both myelinating and ensheathing Schwann cells arise from a common precursor, which is recognized in the rat by the expression of S-100 (a calcium-binding protein) at embryonic day 15 to 16 and the O4 antigen (a sulfatide) 1 to 2 days later (reviewed in Jessen and Mirsky, 1991). Myelination of largest diameter axons begins soon after birth in rats and is followed by myelination of progressively smaller axons just prior to or at the same time as ensheathement of the smallest axons begins, 2 to 3 weeks after birth (Martin and Webster, 1973; Webster et al., 1973). The two subtypes of Schwann cells can be identified by the expression of various components (Mirsky and Jessen, 1984). Myelin-forming Schwann cells express myelin-associated glycoprotein, protein zero, myelin basic protein, peripheral myelin protein 22, and lipids comprising the myelin sheath, including galactocerebroside (Politis et al., 1982; Quarles, 1983; Martini and Schachner, 1986; Jessen et al., 1987a; Lemke and Chao, 1988; Mitchell et al., 1990; Snipes et al., 1992). Ensheathing Schwann cells do not express the myelin proteins, but instead synthesize glial fibrillary acidic protein, neural cell adhesion molecule, nerve growth factor receptor, and the adhesion molecule, L1, and share the expression of galactocerebroside with myelinating Schwann cells (Jessen et al., 1985; Daniloff et al., 1986; Martini and Schachner, 1986; Mirsky et al., 1986; Jessen et al., 1987b). As will be discussed later, expression of these characteristic components is determined by interactions with the axon. Throughout maturity it is generally accepted that both subtypes of Schwann cells remain plastic in their ability to respond to the axon and to redifferentiate into either a myelinating or ensheathing Schwann cell following injury in which axonal contact is first lost, and then reestablished as nerve fibers regenerate (Weinberg and Spencer, 1975; Aguayo et al., 1976a, 1976b).

SCHWANN CELL SUBTYPES: ENSHEATHING AND MYELINATING

The majority of ensheathing Schwann cells are associated with the unmyelinated afferent axons of dorsal root ganglion neurons and the unmyelinated efferent sympathetic axons of autonomic ganglia, such as the superior cervical ganglion. The diameter of these axons generally ranges from 0.4 to 1.0 μm, although unmyelinated axons up to 3.0 μm may sometimes be found (Webster, 1993). Unmyelinated axons are found dispersed among myelinated axons in peripheral nerves and are more often found in closer proximity to small-diameter myelinated axons than to large-diameter myelinated axons. Because the ensheathement provided by nonmyelinating

Schwann cells is not necessary to allow nerve fiber conduction, the function of the ensheathing cell remains to be adequately explained. Myelinating Schwann cells are associated with the larger axons of somatic, sensory, and certain autonomic neurons. In mammals, these axons range in size from 1 to 15 μm. It is generally held that those axons in the peripheral nervous system that attain a diameter of greater than 1 μm have the capacity to induce myelin formation by related Schwann cells (Matthews, 1968; Friede, 1972).

The Ensheathing Schwann Cell

Schwann cells related to unmyelinated nerve fibers provide ensheathement via membranous indentations on the surface of the Schwann cell, which typically provide residence for individual axons in separate troughs (Figure 3-1A). This basic pattern of ensheathment of unmyelinated nerve fibers is substantially modified, however, in several parts of the body (R. Bunge, 1968). In the olfactory nerve, Schwann cells provide common ensheathement for small bundles of axons, often several dozen small diameter axons being contained within one ensheathing trough. Substantially modified ensheathing cells (also known to be of neural crest origin) are found in the myenteric plexus of the gut wall (Jessen and Mirsky, 1983). Here, the nonneuronal cells provide communal ensheathement, both for nerve cell bodies and for nerve processes without separation of these neural elements by intervening cytoplasmic extensions and without the deposition within these neural aggregates of connective tissue. In these regions, a basal lamina surrounds the entire miniature ganglion containing both nerve fibers and nerve cell bodies in a manner reminiscent of the glial limitans of the central nervous system. These glial cells of the enteric plexus contain an intermediate filament with immunocytochemical characteristics of the intermediate filaments within astrocytes (Jessen and Mirsky, 1983). Thus, in their manner of ensheathement, their relationship to basal lamina and their cytoplasmic constituents, they resemble astrocytes of the central nervous system.

The Myelinating Schwann Cell

In the process of forming a segment of myelin, the Schwann cell first elongates along the axon to engulf a domain of axolemma approximately 200 μm in length (Webster, 1993). During this time, the axon is separated away from other axons and a unitary relationship between the Schwann cell and this length of axon is established (Figure 3-2 and 3-3). Direct observations of Schwann cells undertaking myelination in tissue culture indicate that Schwann cells actively compete for axonal domain during this period (Figure 3-3). Evidence has recently been presented that the formation of myelin is accomplished by one lip of the encircling Schwann cell process slipping under the other and pushing itself forward assertively along the axon surface (R. Bunge et al., 1989) (Figure 3-4). As it pushes aside the overlying Schwann cell cytoplasm, the advance of this inner lip draws out a spiral of membrane, which, with compaction, will become the definitive myelin sheath. According to this view, the spiral is formed by the progressive advancement of the inner lip of the Schwann cell over the axon surface rather than by advance of the outer lip of the spiraled Schwann cell cytoplasm over its outer surface.

Upon compaction of the spiraled membranes with myelin sheath maturation, some Schwann cell cytoplasm is retained external to the compacted lamellae as an external cytoplasmic collar, and internally as the internal cytoplasmic collar (Figure 3-1B). Evidence has been presented (reviewed in Griffin et al., 1993) that the retention of cytoplasm in this inner collar is related to its content of myelin-associated glycoprotein. Cytoplasm is also retained in periodic interruptions of membrane compaction within the myelin segment; these regions of cytoplasmic retention are termed Schmidt-Lanterman clefts.

The arrangement of the Schwann cell membranes that form the myelin sheath at the node of Ranvier (Figure 3-2A) is remarkably systematic. The layers of spiraled membrane separate to enclose a small amount of cytoplasm, and this terminal loop is applied to the surface of the axolemma near the node of Ranvier (Figure 3-2B). Successive layers of Schwann cell membrane terminate similarly, the most external layers terminating nearest the nodal region, and the innermost layers of the spiral terminating farthest from the node. This alignment of terminal loops adjacent to the nodal axolemma is termed the perinodal apparatus. The specialized contacts that develop between these loops and the underlying axolemma have been described in detail (Schnapp and Mugnaini, 1975). The cytoplasm within the terminal loops is rich in microtubules, considered important for transport of materials from the external cytoplasmic collar of Schwann cell cytoplasm to the internal collar adjacent to the axon. Nodes of the larger nerve fibers are much more complex than the nodes shown in Figure 3-2, which are formed around an axon only 1.5 μm in diameter. In the paranodal regions of the largest nerve fibers, compact myelin in the paranodal region is fluted,

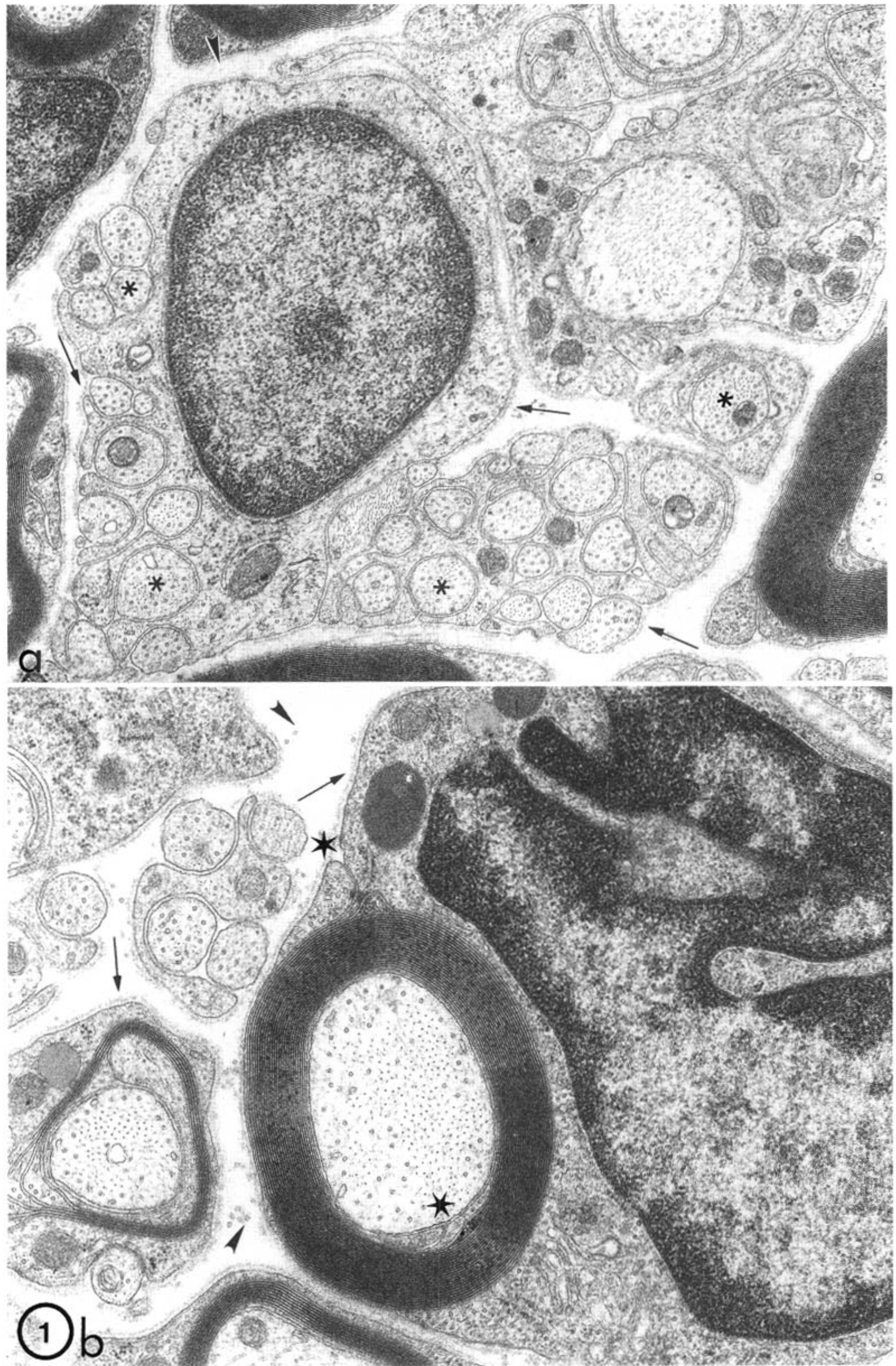


FIG. 3-1. (A) Ensheathing Schwann cell. A Schwann cell with its nucleus is seen in the center of the micrograph performing the function of ensheathement. A number of axons (some marked *) have been individually surrounded by the cytoplasm of this Schwann cell and separated from each other. The entire unit, consisting of a Schwann cell and its related axons, is surrounded by a basal lamina (*arrows*). Collagen fibrils (*arrowheads*) are located outside the basal lamina. Additional ensheathed axons are present to the right. Only the cytoplasmic processes of the Schwann cell and its basal lamina are seen. ×25,000. (B) Myelinating Schwann cell. A Schwann cell with its nucleus is seen in this micrograph performing the function of myelination. The enclosed axon is surrounded by multiple layers of compacted, specialized Schwann cell membrane forming the myelin sheath. The beginning of this spiral at the inner mesaxon and the termination of the spiral at the outer mesaxon are marked (*stars*). Also note the mitochondria, Golgi apparatus, and lysosome in the cytoplasm. The entire unit is surrounded by basal lamina (*arrows*), and collagen fibrils (*arrowheads*) occupy the extracellular space between adjacent myelinated axons. An adjacent smaller axon is at an early stage of myelination. ×25,000. [Courtesy of Dr. Mary Bartlett Bunge.]

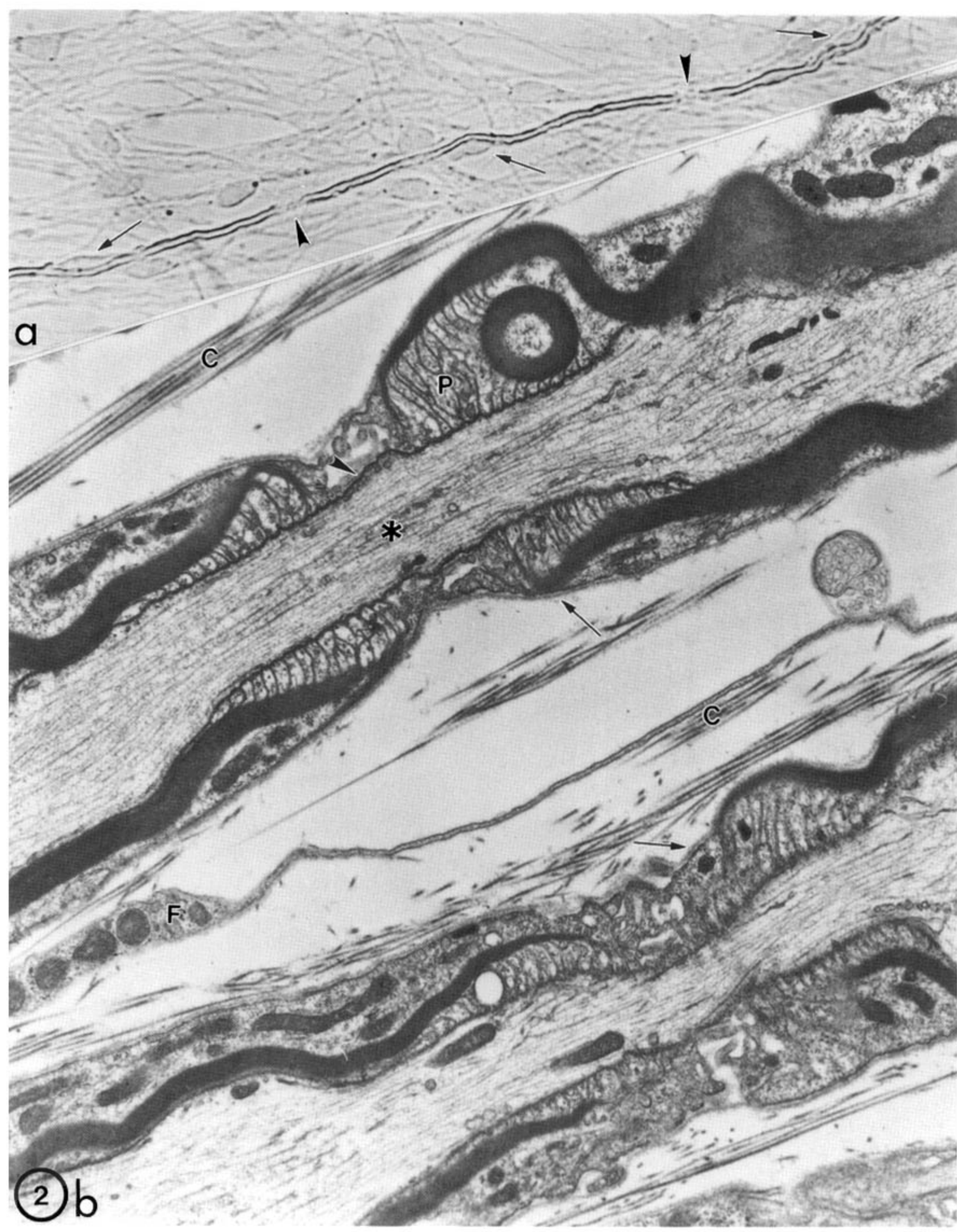


FIG. 3-2. (A) Myelin segments. One myelinated axon from a dorsal root ganglion neuron/Schwann cell coculture is shown. Three Schwann cells (*arrows* point to their nuclei) have positioned themselves along the axon and have elaborated three segments (internodes) of myelin. The nodes of Ranvier are seen as gaps between adjacent myelin segments (*arrowheads*). ×1000. (B) Nodes of Ranvier. By electron microscopy the node of Ranvier is characterized by a constriction of the axon and compaction of cytoskeletal elements (*). The axolemma (*arrowhead*) appears denser and thicker at the node. The myelin membranes seen as paranodal loops (*P*) are less compact, and more cytoplasm is found here than between adjacent myelin lamellae. Schwann cell processes lie over the axon at the node of Ranvier. Basal lamina (*arrows*) is continuous along the entire Schwann cell surface and traverses the nodal region. Longitudinally arranged collagen fibrils (*C*) are present in the extracellular space and a fibroblast (*F*) is seen between the two myelinated axons. ×20,000. [Courtesy of Dr. Eiko Okada.]

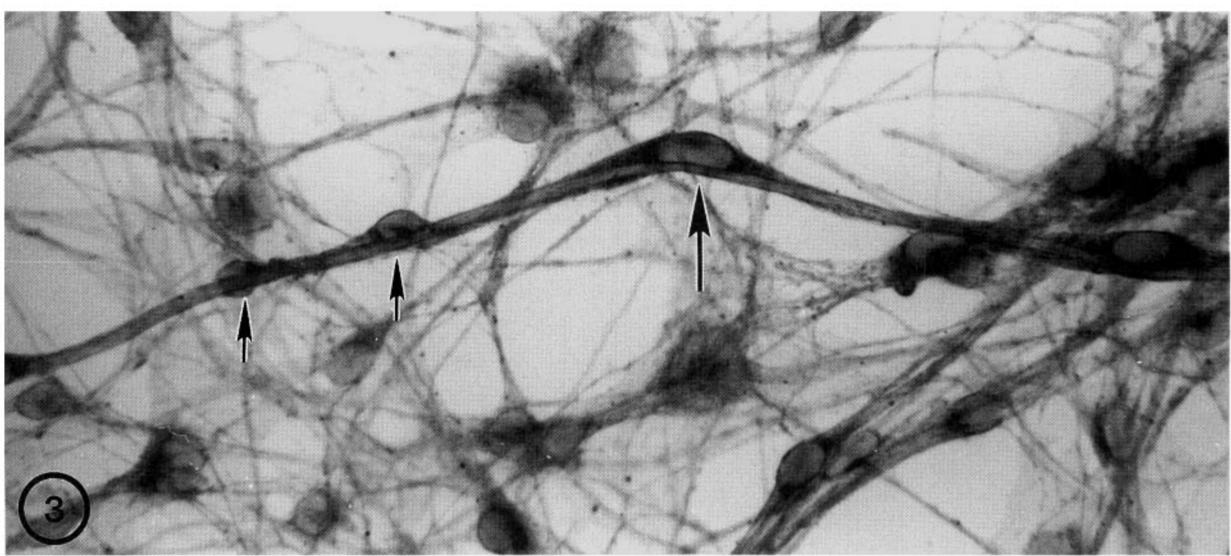


FIG. 3-3. Schwann cells beginning the process of myelination along an axon approximately 1 μm in diameter (which traverses the width of the field) compete for axolemmal space. The Schwann cell (*large arrow*) is laying claim to a substantial length of the axon preparatory to initiating the deposition of myelin, and in the process is pushing aside two adjacent Schwann cells (*small arrows*). It is not clear whether these redundant Schwann cells survive and find alternate function, or die. From a culture containing sensory neurons to which a purified population of Schwann cells was added. ×500.

and is interspersed with substantial regions of retained cytoplasm (Thomas et al., 1993).

The end result of the process of myelination in the peripheral nervous system is the deposition of a segment of spiraled compacted membrane, which occupies an axon length between 250 and 1000 μm and is separated from the adjacent segment by a node of Ranvier (Figures 3-1B and 3-2). With the deposition of these segments, the underlying axolemma is modified so that certain of the sodium channels that characterize excitable tissues are concentrated in the region exposed to the external environment at the node of Ranvier (Chiu, 1993). It is the concentration of these ion channels at the nodes that provides for focal current generation and leads to saltatory conduction. Details of the physiology of the myelinated axon and the specific function of the components of the node of Ranvier are discussed in Chapter 40. The manner in which the structure of the axon enclosed within myelinating Schwann cells is modified by this ensheathement is discussed in detail in Chapter 51.

The Ultrastructure of Schwann Cells

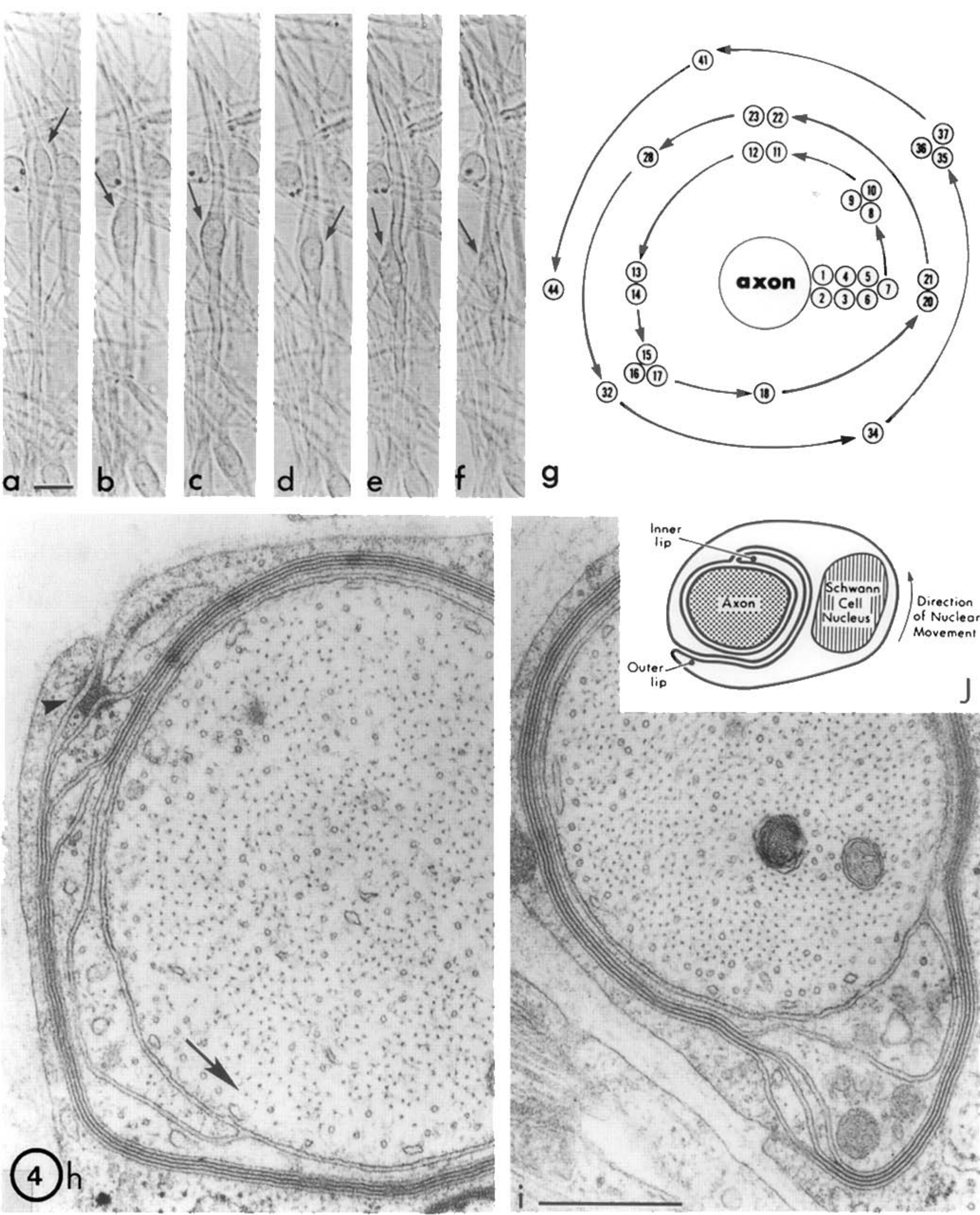
After reaching maturity, Schwann cells typically have a small ovoid nucleus containing a substantial amount of condensed chromatin, often clumped both at the nuclear periphery and near the center of the nucleus where one or more nucleoli are typically seen. Schwann cell cytoplasm is dense, containing both free and membrane-associated ribosomes, many rounded mitochondria, the Golgi apparatus, and a centromere found in the perinuclear region. The cytoplasm also contains lysosomes, vesicular bodies, and peroxisomes approximately 0.2 μm in diameter. Schwann cells contain microtubules of standard dimension and intermediate filaments. These and other cytoskeletal elements of the Schwann cell are considered in detail in Chapter 42. The nuclear and cytoplasmic characteristics of myelinating and nonmyelinating Schwann cells are similar (Figure 3-1A, B).

SCHWANN CELL INTERACTIONS WITH AXONS AND FIBROBLASTS

The expression of the higher differentiated functions of the Schwann cell is regulated by contact with the axon and, in some instances, enhanced by interactions with fibroblasts. Four aspects of Schwann cell function are known to be directly controlled by axonal contact: differentiation into an ensheathing or myelinating cell, myelin segment length, proliferation, and basal lamina production.

Axons Determine Whether a Schwann Cell Will Ensheathe or Myelinate

It has long been recognized that axonal properties determine whether Schwann cells will myelinate or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ensheathe axons. This was definitively shown by cross-anastomosing myelinated with nonmyelinated nerves and observing that the resident ensheathing Schwann cells of nonmyelinated nerve stumps differentiated into myelinating Schwann cells when interacting with axons originating from myelinated nerves (Weinberg and Spencer, 1975; Aguayo et al., 1976a, 1976b). Loss of axonal contact causes myelinating Schwann cells to stop expressing or to reduce the level of expression of myelin-associated glycoprotein, protein zero, myelin basic protein, and peripheral myelin protein 22, and to express glial fibrillary acidic protein, neural cell adhesion molecule, and nerve growth factor receptor (Politis et al., 1982; Poduslo, 1984; Jessen et al., 1987a; Tanuichi et al., 1988; Trapp et al., 1988; Mitchell et al., 1990; Fan and Gelman, 1992; Snipes et al., 1992). The expression of the sulfatide O4 and galactocerebroside are also downregulated upon loss of axonal contact in both myelinating and ensheathing Schwann cells, whereas S-100 expression remains constant in both Schwann cell types (Jessen et al., 1985; Jessen et al., 1987b). Thus, signals from axons are important determinants of phenotypic-specific gene expression in Schwann cells; these signals, however, have not been identified. Voyvodic (1989) has shown that increasing the target size of sympathetic axons, which are normally unmyelinated, will cause axon diameter to increase; these enlarged axons then become myelinated.

Axon Diameter Determines the Length of the Myelin Segment

Axons and Schwann cells also interact to influence axonal diameter; this, in turn, influences myelin internode length. The evidence that maturation of axons to full diameter depends on the presence of normal Schwann cells and the formation of myelin sheaths has been recently reviewed (Aguayo and Bray, 1984; Hoffman and Griffin, 1993). Axonal diameter is decreased in cases in which myelin sheaths are lost, as in the naked axons of the spinal roots of the mutant dystrophic mouse (Aguayo et al., 1979). Remyelination increases axon diameters of demyelinated axons and ensheathement increases axon diameter in dystrophic mice. Transplants of normal Schwann cells have the capability of increasing the abnormally small diameter of axons in the mouse mutant *trembler* (de Waegh et al., 1992). Tissue culture experiments have provided direct demonstration that axon diameters are increased in areas of cultures which contain Schwann cell ensheathement as compared to adjacent regions in the same culture where the axons are not ensheathed (Windebank et al., 1985).

Axons Are Mitogenic for Schwann Cells

The influence of axon contact on Schwann cell numbers has been established *in vitro* and *in vivo* (for review, see DeVries, 1993). The number of Schwann cells produced during embryogenesis can be decreased by decreasing the number of nerve fibers within peripheral nerve trunks (Aguayo et al., 1976c). In tissue culture, it is possible to directly demonstrate that axon contact influences Schwann cell proliferation and, moreover, that direct contact between the axon and Schwann cell is required (Wood and Bunge, 1975). This *in vitro* work has established that the mitogenic agent is a component of the axon surface (Salzer and Bunge, 1980). Evidence has recently been provided that the mitogenic activity expressed on rat cultured sensory neurons is associated with a heparin sulfate proteoglycan component of the neurites (Ratner et al., 1985). Axolemmal preparations derived from adult central white matter and adult sciatic nerve are also mitogenic for Schwann cells (DeVries et al., 1982; Sobue et al., 1983). DeVries has recently compared the neuritic and axolemmal mitogens and has presented evidence suggesting that these are different molecules, per-

FIG. 3-4. Evidence that the circumnavigation of the axon by the Schwann cell during myelination reflects advance of the inner rather than the outer end of the membrane spiral. *a–g* document the nuclear (*small arrows*) movement that occurred during a 44-hour observation period; the positions at 6 (*a, east*), 15 (*b, west*), 18 (*c, below the axon*), 20 (*d, east*), 28 (*e, west*), and 44 (*f, west*) hour of observation are shown. *a–e* illustrate $1^1/_2$ rotations (*east-west-east-west*) and *f* shows the results of a further rotation (*west-east-west*). Myelin formed during the observation period; the distinctive refractivity of myelin is apparent in *e* and *f*. In all, the nucleus circumnavigated the axon $2^1/_2$ times, illustrating that the nucleus can move substantially in early myelination and systematically in one direction. The direction in which the inner Schwann cell lip is pointed (counterclockwise; *h, arrow*) corresponds to the direction of nuclear circumnavigation. *i* shows a level of the internode ≈100 μm away from that illustrated in *h*. (*a–f*) Living culture. Bars: (*a–f*) 10 μm; (*h, i*) 0.5 μm. The inset (*J*) summarizes the concept. It shows a transversely sectioned, myelinating Schwann cell in the early phases of mesaxon formation preceding myelination. Circumnavigation of the axon by the Schwann cell nucleus is observed during early myelinogenesis to be in the direction of the inner lip of the spiraling Schwann cell process and opposite the direction of the outer lip. [From Bunge et al. (1989), with permission of The Rockefeller University Press.]

haps changing with the maturation state of the axon. He has also summarized the data available on the intracellular and molecular events which occur subsequent to mitogenic stimulation (DeVries, 1993).

Several methods are now available which allow separation of Schwann cell populations from both developing and adult peripheral nerve (Brockes et al., 1979; Morrissey et al., 1991). This has allowed a general analysis of the agents capable of inducing proliferation of Schwann cells that are not in contact with axons. Brockes and coworkers (Brockes et al., 1980; Lemke and Brockes, 1984) first identified a potent mitogen from bovine pituitary gland, a peptide termed *glial growth factor,* which now has been cloned and sequenced (Marchionni et al., 1993). There are three forms of the growth factor, which are products of alternative splicing of a single mRNA. The gene that encodes human glial growth factor also encodes several ligands known as *heregulins* for the $p185^{erbB2}$ receptor tyrosine kinase, which is structurally related to the epidermal growth factor receptor (Holmes et al., 1992). The homologous genes in chicken and rat encode the acetylcholine receptor inducing activity and *neu* differentiation factor, respectively. These three related factors may play key roles in the development and regeneration of the nervous system (Wen et al., 1992; Falls et al., 1993; Lemke, 1993; Reynolds and Woolf, 1993).

It is now known from the work of Davis and Stroobant (1990) (among others) that additional growth factors for Schwann cells include transforming growth factors β1 and β2. Acidic and basic fibroblast growth factor, and several forms of platelet derived growth factor are effective mitogens when used in combination with agents that raise intracellular cyclic amp levels. DeVries (1993) has recently reviewed other agents that influence the Schwann cell's responsiveness to mitogens. These molecules include proteases, extracellular matrix components, and products of myelin degradation.

Axons Induce Schwann Cells to Form Basal Lamina

An extensive series of studies over the past decade has established that axonal contact directly influences the deposition of basal lamina by the Schwann cell. This work has also demonstrated that basal lamina production is essential for Schwann cell function (for review see M. Bunge, 1993; M. Bunge et al., 1989b). As has been discussed above, this basal lamina deposition is characteristic of each axon-Schwann cell unit formed during the development of peripheral nerve. The contributions of axon-Schwann cell contact and of Schwann cell-fibroblast interaction in the deposition of the basal lamina and other connective tissue components of the peripheral nerve have been studied in a series of tissue culture experiments in which these three cell types have been grown in various combinations. When grown in isolation, Schwann cells express both laminin and proteoglycan on their surface, but do not organize a basal lamina (Carey et al., 1983; Cornbrooks et al., 1983; Eldridge et al., 1989). If Schwann cells are cocultured with neurons in a fibroblast-free system, it can be demonstrated that basal lamina is formed around each axon-Schwann cell unit, and a small number of collagenous fibrils are deposited (M. Bunge et al., 1980; M. Bunge et al., 1982; Eldridge et al., 1987). This demonstrates that the Schwann cell is able to generate basal lamina components when in contact with axons without the aid of fibroblasts. If neurons are removed from a culture of Schwann cells and neurons after a substantial basal lamina has been deposited, the basal lamina is retained. If this basal lamina is removed by enzymatic digestion, the orphan Schwann cell will not form a new basal lamina unless contact with axons is restored (M. Bunge et al., 1982).

Fibroblasts Enhance Schwann Cell Function

Recent experiments have shown, however, that the fibroblast is very much involved in cooperating with the Schwann cell in depositing basal lamina around the axon-Schwann cell units that characterize nonmyelinated nerve fibers (Obremski et al., 1993a). In cultures of sympathetic neurons whose axons are not normally myelinated, ensheathement of the axon is incomplete unless fibroblasts are present. In the presence of fibroblasts ensheathement is brought to completion and a full basal lamina is deposited around each axon-Schwann cell unit. It has further been demonstrated that this fibroblast influence on Schwann cell behavior can be mediated by materials released from fibroblasts, and does not require immediate contact between these two cells (Obremski et al., 1993b). Biochemical analyses of these types of preparation have shown that Schwann cells are capable of secreting types I, III, IV, and V collagen, as well as laminin and proteoglycans (Carey et al., 1983; Eldridge et al., 1989). Type IV collagen and laminin, the major components of basal lamina, are prominent products of Schwann cells studied in culture when in contact with axons (M. Bunge, 1993).

SCHWANN CELL FUNCTION IN INJURY AND REPAIR

Schwann Cell Reactions in Wallerian Degeneration

The remarkable versatility of the Schwann cell is well illustrated in the responses that it undergoes during the process of axonal breakdown known as Wallerian degeneration (for review see Griffin et al., 1993). When a peripheral nerve is transected there is a prompt breakdown of the amputated axon distal to the cut over a period of 12 to 24 hours. Concomitant with this the myelin sheath also begins to break down. As the myelin and axon disintegrate, both the resident Schwann cells (Figure 3-5) as well as hematogenous macrophages (which are recruited into the nerve by the degenerative process) are involved in the removal of axonal and myelin debris (Stoll et al., 1989). Shortly after the beginning of this breakdown, many of the Schwann cells in a nerve undergoing Wallerian degeneration enter the cell cycle. The Schwann cell division begins 3 to 4 days after a nerve is cut and continues for the subsequent 3 weeks after injury. Griffin and his colleagues (1990) have presented evidence that during this period of cell proliferation there is a release of diffusible factors, which influence not only the Schwann cells involved in the process of myelin breakdown but also nearby nonmyelinating Schwann cells, endoneurial fibroblasts, and perineurial cells. Within several days after nerve injury, the Schwann cells distal to the injury change their synthetic program, with a marked reduction in synthesis in myelin constituents. Thus, the Schwann cells lose a number of their phenotypic markers that are associated with myelin maintenance, including the expression of protein zero, galactocerebroside, and myelin basic protein.

Concomitant with this loss of phenotypic markers, Schwann cells begin synthesis of a number of trophic factors, including nerve growth factor (Heumann et al., 1987), brain-derived neurotrophic factor (Acheson et al., 1991), neurotrophin 3 (Schechterson and Bothwell, 1992), and platelet-derived

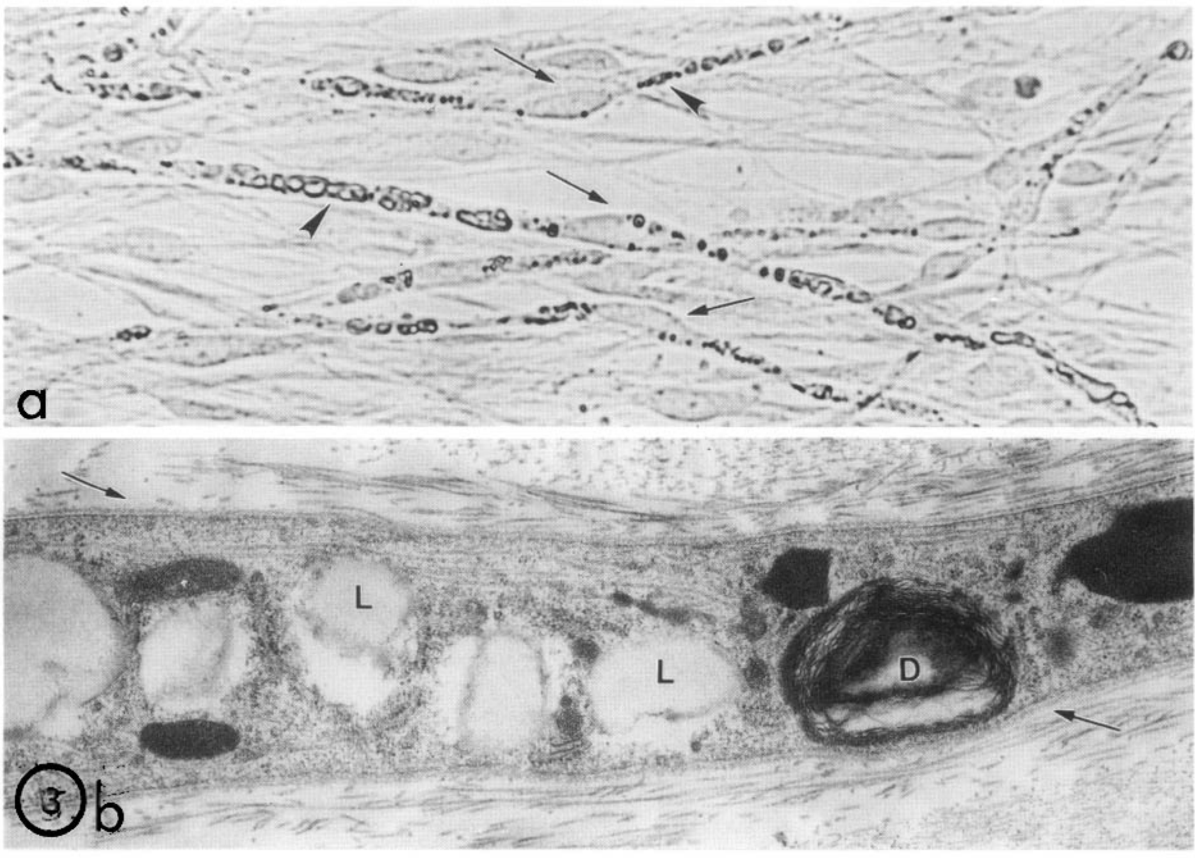

FIG. 3-5. (**A**) Myelinating axons undergoing Wallerian degeneration. Several myelin-forming Schwann cells (*arrows* point to location of their nuclei) in a dorsal root ganglion neuron-Schwann cell coculture 3 days after severing axons show fragmentation of their myelin sheaths (*arrowheads*). Schwann cells remain elongated within the basal lamina tube (see below) although the axon has degenerated. ×1000. (**B**) A Schwann cell 6 days after initiation of wallerian degeneration. The cytoplasm is filled with myelin breakdown products, including lipid droplets (*L*) and membranous fragments of myelin debris (*D*). The Schwann cell is elongated and basal lamina (*arrows*) is retained along its surface. ×25,000.

growth factor (Eccleston et al., 1990). These factors are downregulated with Schwann cell differentiation and upregulated in Wallerian degeneration. An exception is the trophic factor ciliary neurotrophic factor, which is upregulated in Schwann cells during full differentiation (Rende et al., 1992). The levels of ciliary neurotrophic factor in Schwann cells are decreased during Wallerian degeneration (Friedman et al., 1992).

Role of Schwann Cells in Regeneration

The responses of the Schwann cells in the process of Wallerian degeneration described in the preceding section are an important prelude to the Schwann cell's role in regeneration (for review see R. Bunge and Hopkins, 1991). Because the connective tissue components of the peripheral nerve (including the tubular basal lamina present around each axon-Schwann cell unit) are preserved within the nerve trunk distal to an injury, those Schwann cells that have been involved in the phagocytosis and digestion of myelin retain residence within endoneurial tubes and are in a position to await the arrival and then to assist the regrowth of axons. There has been an ongoing debate regarding the importance of the Schwann cell vis-à-vis the extracellular matrix components of the peripheral nerve, in fostering the process of regeneration in the peripheral nervous system (for reviews see Kromer and Cornbrooks, 1987; M. Bunge et al., 1989b; for discussion see Kleitman et al., 1988; Paino and Bunge, 1991). It now seems clear that, whereas the components of the extracellular matrix, which form the basal lamina retained in the distal nerve stump in Wallerian degeneration, are able to promote some degree of axonal regrowth, this regrowth is much more effective if these basal lamina tubes are occupied by Schwann cells (Ide et al., 1983; Ard et al., 1987; Bixby et al., 1988; Kleitman et al., 1988; Hopkins and Bunge, 1991). When axons are allowed to grow into peripheral nerves in which the Schwann cells have been removed, the process of regrowth is less vigorous and, as the axons grow, they are accompanied by Schwann cells migrating in from the proximal stump of the cut nerve (Sketelj et al., 1989). In the peripheral nerve of the rodent the process is rapid, and remyelination of the regrowing axons in the distal stump occurs within 3 weeks after injury. It should be noted that Schwann cells resident within peripheral nerves subjected to myelin loss (demyelination) are capable of promptly replacing the lost myelin segments (Griffin et al., 1990). Because there is no subsequent growth of the remyelinated nerve, these segments will generally remain shorter than those which have been retained from the neonatal period, thus allowing recognition of the remyelinated nerve fiber. Schwann cells isolated from adult peripheral nerve and expanded in tissue culture have been demonstrated to be effective in promoting peripheral nerve regeneration when aligned within a guidance channel and placed between cut ends of the sciatic nerve of the rat (Guenard et al., 1992).

CONCLUDING REMARKS

It has now been shown in a number of situations that purified populations of Schwann cells taken from the peripheral nervous system have the capability of engendering considerable axonal regeneration within the central nervous system (Kromer and Cornbrooks, 1985; Paino and Bunge, 1991; Xu et al., 1994). Because we can now separate the Schwann cell population from the other cellular and matrix components of the adult peripheral nervous system (Morrissey et al., 1991; Rutkowski et al., 1992), it is possible to isolate Schwann cell populations, to expand their numbers, and to use them to construct cellular protheses to influence the regeneration of both central and peripheral neural tissues (Guenard et al., 1992; Levi et al., 1994; Xu et al., 1994).

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4 Ependymoglia and ependymoglia-like cells

ANDREAS REICHENBACH AND STEPHEN R. ROBINSON

DEFINITION, OCCURRENCE, AND PHYLOGENY OF EPENDYMOGLIA

This chapter argues for the view that vertebrate radial glia, ependymal cells, and supporting cells of sensory epithelia share a common evolutionary origin, and that they are functionally and structurally homologous. The Greek term *ependyma* (επι=upon; ενδυμα=garment) was introduced to designate the cell layer that lines the ventricular spaces of the vertebrate brain. Thus, ependymoglial cells are defined here as glial cells that extend one of their processes (or abut with their soma) to the lumen of the ventricular system, and contribute to the apical surface of neural tissue (tissue that is epithelial in deuterostomes, to which vertebrates belong) (see Table 4-1). Typically such cells are bipolar, and their processes may span the entire width of the neural epithelium (*radial glia*). Some types of ependymoglia (e.g., fetal radial glia, Müller cells) have been classified as "astrocytes" by some authors, but, as will be seen in this chapter, they possess many features not shared by astrocytes. Conversely, astrocytes with radially aligned processes (e.g., Bergmann glia) have sometimes been included with ependymoglia. These cells, however, do not establish contact with the apical surface of neural tissue, and they lack many of the features that characterize ependymoglia.

Ependymoglia-like cells are found in the central nervous system of invertebrates that have epithelial nervous systems (i.e., deuterostomes—with the exception of chaetognatha, some turbellarians, and some oligochaetes) and in the sensory epithelia of most animal phyla. These cells abut a fluid- or mucus-filled lumen, and have a bipolar morphology. Such cells do not occur in nonepithelial nervous tissues, where both an apical surface and a fluid-filled lumen are missing. They are absent from the peripheral ganglia of vertebrates (which arise from migrating neural crest cells) and from the ganglionic central nervous system of most protostomes where the epithelial character of the tissue is lost in early development.

Since ependymoglia-like cells in invertebrates provide insights into the possible phylogenetic origins of ependymoglia, we begin with a brief discussion of these cells, followed by a review of ependymoglia-like cells in placode-derived sensory epithelia, and, finally, we discuss "classical" ependymoglia. The last part of the chapter deals with general types and possible growth mechanisms of ependymoglial cell processes, the ontogeny of ependymoglial cells, and the relationship of ependymoglial cells to astrocytes. Several excellent reviews of ependymoglia have already been published (e.g., Leonhardt, 1980; Bruni et al., 1985).

Phylogenetic Origin of Ependymoglia-like Cells

In coelenterates, neuronal cells are scattered throughout the epidermal tissue, but in some medusae rather elaborated sensory epithelia (Pearse and Pearse, 1978) and ganglia (Horridge and McKay, 1962) occur. Even the most primitive deuterostomes have an epithelial central nervous system that is linked to epithelial sensory fields (cf. Bullock and Horridge, 1976) (see Figure 4-1). In these animals (e.g., starfish) the cell-dense apical zone of the nervous system forms part of the body wall and is separated by a thin cuticle from the seawater. Sensory neurons in this zone direct their receptive processes into this environment, and deliver signals to multipolar ganglion cells that distribute this information laterally via a nerve fiber layer of considerable thickness, which forms the inner zone of the nervous tissue. "Epidermal" or "supporting" cells compose a third cellular element of this system, and have long, radial processes that span the entire thickness of the tissue up to the basal lamina beneath the underlying mesenchyma (Meyer, 1906; Bargmann et al., 1962; Rehkämper et al., 1987). These cells closely resemble vertebrate ependymoglia (see also Figure 4-3A).

There are good arguments to support the view that ancestral chordates were derived from larval forms of primitive deuterostomes (i.e., are of neo-

TABLE 4-1. *Types of Ependymoglia and Related Cells Found in Vertebrates*

Cell Type	Tissue
Ependymoglia (radial glia)	
Tanycytes	Brain and spinal cord
Tanycyte-like (e.g., coronet) cells	Circumventricular organs
Support cells	Parietal/pineal organ
Müller cells	Retina (lateral eye)
Ependymocytes	
Ciliated ependymocytes	Lining of ventricles
Choroid plexus epithelial cells	Circumventricular organs
Lens cells	Parietal eye
Pigment epithelial cells	Lateral eye
Ependymoglia-like cells	
Supportive cells	Ampullary electroreceptor
Support cells	Neuromast organ
Support cells	Vestibular organ
Support (pillar, border, Deiters', Hensen's and Claudius') cells	Organ of Corti
Supporting (sustenacular) cells	Olfactory epithelium
Supporting (light) cells (?)	Taste bud

tenic origin: e.g., Garstang, 1928; Bone, 1981; Lacalli, 1990; Crowther and Whittaker, 1992). The nerves of such larvae (as in the related lophophorates: Figure 4-2B) are mostly intraepithelial, and are ensheathed by ependymoglia-like cells. The ciliary bands of these larvae (Figure 4-2A) are thought to have been the precursors of the vertebrate neural plate and tube (e.g., Lacalli et al., 1990); their nervous system consists of the same principal types of cellular elements as that of the adult animals. Thus, the "prototype" neural tube was probably an "enfolded" or "enrolled" version of the epithelial nervous tissue shown in Figure 4-1, and was composed of sensory cells (cerebrospinal fluid contact neurons: Vigh et al., 1969), multipolar ganglion cells, and ependymoglia-like cells. Even in the earliest vertebrates, this kind of central nervous system seems to have been interconnected with ectodermal sensory epithelia, consisting of receptor neurons and "supporting" cells (Northcutt and Gans, 1983).

TYPES OF EPENDYMOGLIA-LIKE CELLS

Figure 4-3 provides a survey on common ultrastructural features of ependymoglial and ependymoglia-like cell types in different nervous tissues of various animal species. All these cells have a bipolar shape and span the tissue between their apical pole that sends microvilli and/or cilia into a fluid-filled space, and their endfoot abutting the basal lamina. All these cells contain intermediate filaments that consist of specific proteins (cf. also Eng and Lee, Chapter 43, this volume). There are, however, other features specific for each particular radial glial cell; moreover, some ependymoglial cell types show different (non-radial) morphologies or extreme adaptations to special functional tasks.

Ependymoglia-Like Cells of Placodal Origin: Supporting/Sustentacular Cells of (Peripheral) Sensory Epithelia

In vertebrates, most sensory epithelia are derived from surface ectodermal placodes, and they can be specialized for mechanoperception (stato-acoustic systems with organs of Corti and vestibular organs; neuromast or lateral line organs in fish and amphibia), electroception (ampullary and tuberous receptor organs in fish and urodeles), or olfaction (nasal olfactory epithelium). Neurons in these sensory epithelia project their receptive organelles (generally a cilium) into a fluid- or mucous-filled lumen, and are enclosed by the processes of support cells. Since these support cells closely resemble ependymoglia, they will be briefly reviewed here, even though they are not true ependymoglia since they do not line the ventricular surfaces.

In electroceptive ampullary organs (e.g., Jørgensen et al., 1972) and most mechanoceptive epithelia (Lindeman, 1969), the apical poles of supporting cells fuse with those of adjacent cells to form an apical "membrane". Adjacent cells are generally joined by tight junctions. Most of these cells possess short microvilli that project into the lumen, and numerous mitochondria and glycogen granules are found near the bases of the microvilli, indicating that these are sites of intense metabolic exchange (Figures 3D, 4A). Such ependymoglia-like cells are generally columnar and span the entire thickness of the sensory structure. Their basal endfeet abut the underlying basal lamina, and surround the axons passing through the basal lamina.

The organ of Corti is unique among the mechanoreceptive epithelia because it contains so many distinct and specialized types of supporting cells, all of which are derived from columnar cells in the epithelium of the otic vesicle. Each type of cell extends from the basilar membrane to the free surface of the organ of Corti where their processes unite to form an apical (reticular) membrane. All of the spaces contacted by these cells are filled with endolymph. One type, the *pillar cell,* has a cement-like basal cone from which tonofilaments extend up to the apical

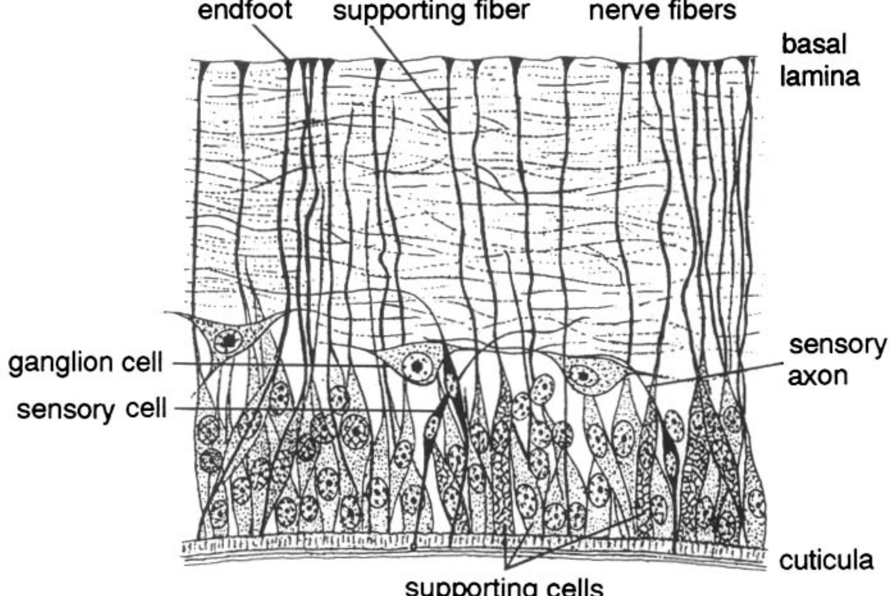


FIG. 4-1. Cross-section through the radial nerve of a starfish (*Asterias rubens*). Directly beneath the cuticula lies the so-called nuclear zone containing the somata of sensory cells, ganglion cells, and supporting cells, which later send long processes ("supporting fibers") through the entire thickness of the epithelium. The conical endfeet of these processes abut the basal lamina. [Redrawn after Figures 4 and 12 in Meyer (1906).]

end of the cell. Some of the tonofilaments are tubular (28 nm outer diameter), while others are microfilamentous (6 nm diameter), and together they form a regular, tightly packed array (Angelborg and Engström, 1972). A second cell type, *Deiters' cells* (outer phalangeal cells), have similar tonofilaments and support the outer hair cells. A third type, *border cells,* are located at the medial side of the organ. They enclose the inner hair cells. Laterally, *Hensen's cells* and *Claudius' cells* form a columnar epithelium that decreases in height with increasing laterality. Vimentin (considered as a marker of central nervous system ependymoglia) is present in two types of supporting cells in the mammalian organ of Corti: Deiters' cells and inner pillar cells (Oesterle et al., 1990).

Supporting ("sustentacular") cells in olfactory epithelia (Figures 4-3C and 4-16A) resemble those of mechanosensory epithelia, except that they contain pigment granules and secretory vesicles, and large Golgi complexes in the apical cytoplasm, indicating

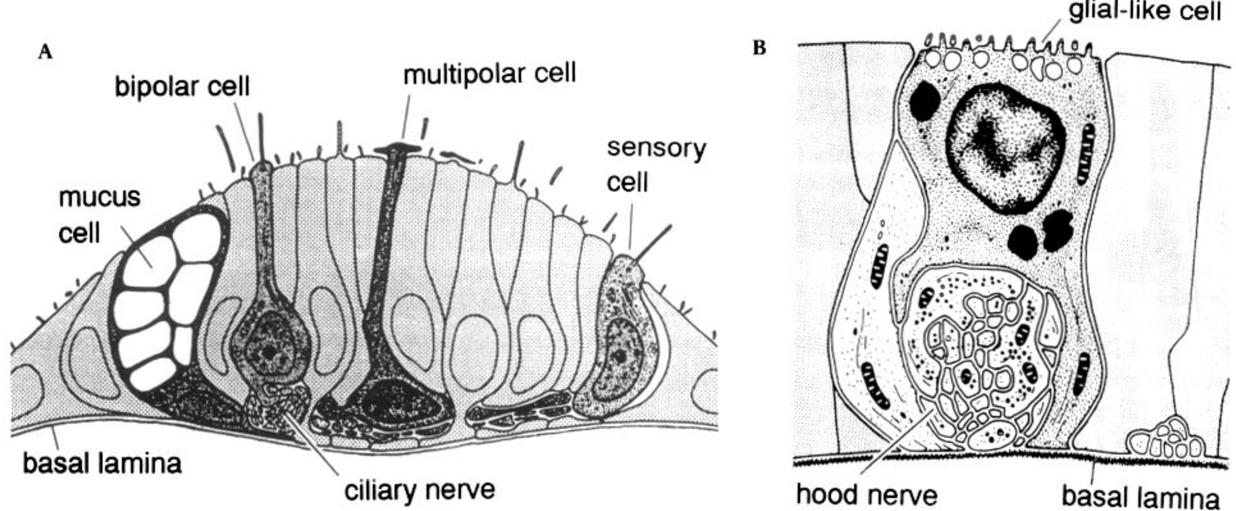


FIG. 4-2. "Supporting cells" of primitive larval nervous systems. **(A)** Transverse section through the ciliary band of the bipinnaria larva of a starfish (Deuterostomia). Three different types of neurons ("sensory," "bipolar," and "multipolar" cells; most probably, the ventricular processes of all three types have sensory functions) are embedded between ciliated supporting cells (*gray stippling*). **(B)** Epithelium of a lophophorate actinotroch larva (Protostomia, but closely related to ancestral stem forms leading to Deuterostomia). The primary hood nerve is ensheathed by a glial-like capsular cell that bears great similarity to supporting cells of vertebrates. [From Lacalli (1990), with permission.]

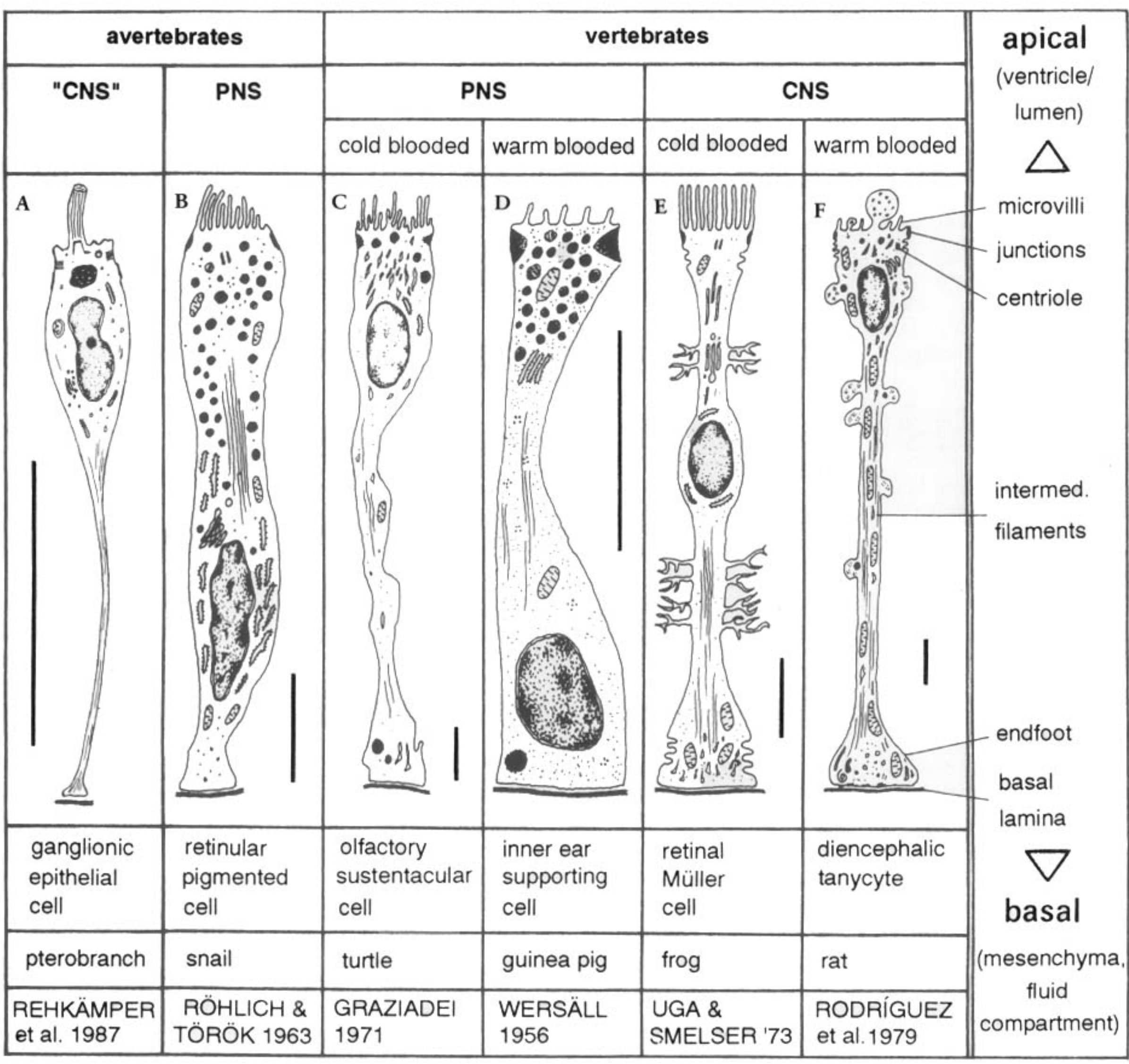
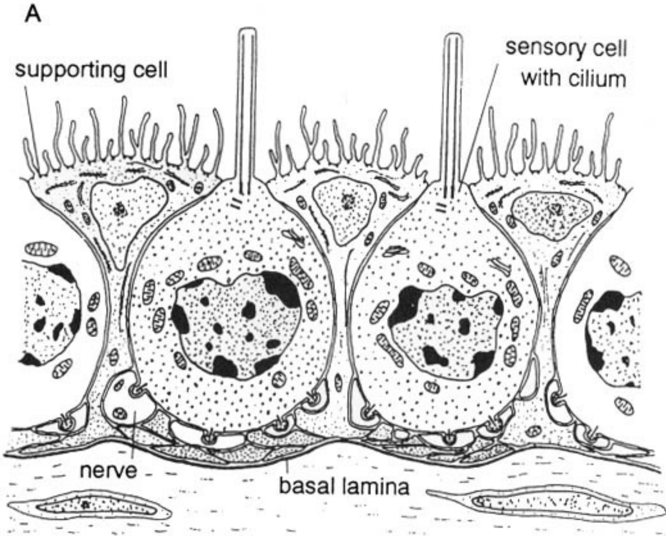


FIG. 4-3. Ultrastructure of various radial glial (or "supporting") cell types in peripheral (*PNS*) and central (*CNS*) nervous tissues of invertebrates and both cold- and warm-blooded vertebrates. The common features of these cell types are given at the right margin. The apical pole of all cells abuts a fluid- (or mucus-) filled space that might be the seawater environment in invertebrates, or the cerebrospinal fluid within the central canal in vertebrates. This pole is characterized by microvilli (and/or cilia), and by sophisticated apicolateral cell coupling. The opposite pole of the cells forms an endfoot at the basal lamina that demarcates the neuroepithelium from underlying mesenchyma. The processes of these cells may be rather long (bar = 20 μm), and contain particular filaments. (The cells were redrawn from the sources given at the bottom line.)

a secretory function. Secretions from their apical surface are thought to contribute to the mucous covering of the epithelium (Getchell et al., 1984), at least in lower vertebrates.

In contrast to the above-mentioned organs, which all arise from ectodermal placodes, gustatory organs (taste buds) develop from placodes within the endoderm. In heterothermic vertebrates, taste buds resemble neuromasts, and contain ependymoglia-like supporting cells (Figure 4-4C). In mammals, a type of supporting cell ("light cell") with ependymoglial features is also present, but the identity of these cells is uncertain. Some authors claim that light cells are actually immature taste receptor neurons, while others claim that they are a nonneuronal support cell and derive from a separate lineage (e.g., Farbman, 1980).

Similar sensory epithelia are found in invertebrate species. In most of these species, the eyes arise from



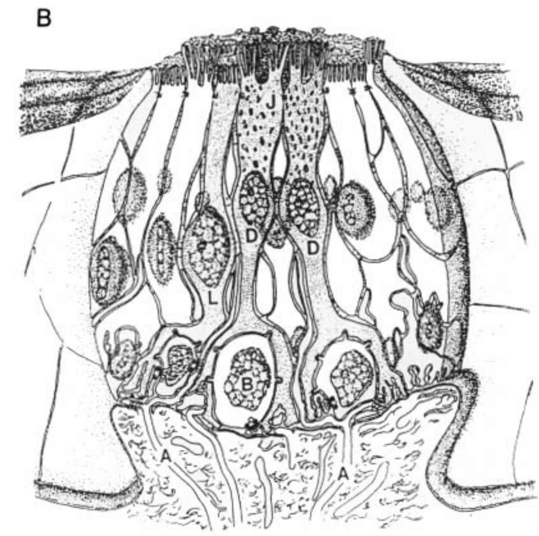


FIG. 4-4. Supporting cells. (A) Electrosensory epithelium in the Lorenzinian ampullary organ of a fish (*Polyodon spathula*). [From Jørgensen et al. (1972), with permission.] (B) A taste bud from *Necturus maculosus*. [From Roper (1989), with permission.] Type I, or dark cells (*D*) are receptor cells, whereas type II, or light cells (*L*) might be supporting cells. All cells are interconnected by apical junctional complexes (*J*). Basal cells (*B*) form synapses with other taste cells and with gustatory axons (*A*), suggesting that they may be a form in interneuron in the taste bud.

ectodermal anlagen outside the central nervous system. Supporting cells of such eyes mostly contain pigment granula (Figure 4-3B).

Ependymoglia of Neural Plate Origin: Central Nervous System Radial Glia and Ependymocytes

"Epithelial" Ependymocytes. In tunicate larvae, thought to be closely related to ancestral vertebrates, most of the neural tube consists of a simple arrangement of "epithelial" ependymocytes (Figure 4-5A). The lumen of their neural tube is enclosed by four (dorsal, ventral, and two lateral) rows of primitive ciliated ependymal cells (Crowther and Whittaker, 1992). There is basolateral contact to the two longitudinal axon bundles.

Within the central nervous system of vertebrates, there is at least one organ that consists of a homogeneous epithelium. The choroid plexus is found in all vertebrate taxa, including cyclostomes. The four choroid plexuses arise from invaginations of the single-layered roof plate; the early anlagen consist of glycogen-rich cells (cf. Dohrmann, 1970). The mature plexus appears as a convoluted vascular membrane whose surface is greatly increased by villi. In humans, the total surface area of the choroid plexuses has been estimated to be about 213 cm^2 (Voetmann, 1949). Each choroidal villus is composed of a single layer of cuboidal ependymocytes resting on a basal lamina, a layer of interposed connective tissue elements, and a blood vessel (mostly a thick capillary) beneath (Figure 4-5B).

The apical membrane of the ependymal cells typically extends large numbers of long microvilli, and a thin central bundle of kinocilia of the $9 \times 2 + 2$ type (the number of which varies with age and species) (Dohrmann, 1970). The lateral membranes are closely approximated, and tight junctions are established near the ventricular surface (Brightman and Reese, 1969). Numerous cristate mitochondria are dispersed throughout the cytoplasm and are often seen in close approximation to components of the abundant rough endoplasmic reticulum. The basal plasma membrane, which rests on the basal lamina, is elaborately infolded. Together with the apical microvilli, these basal infoldings characterize the choroid plexus as a transport epithelium. The choroid plexuses are responsible for most of the cerebrospinal fluid production by means of their apical sodium-potassium pumps that transport, into the lumen, an excess of sodium ions, which drag along water and soluble molecules (e.g., Wright et al., 1977). This function is subject to neuroendocrine regulatory mechanisms (cf. Nilsson et al., 1992).

There are obvious structural similarities between the ependymocytes of the choroid plexus and those of another epitheloid ependyma, the *retinal pigment epithelium* (Figure 4-5C). It separates a cavity, the subretinal space (a vestige of the embryonic optic ventricle), from the blood within the choriocapil-

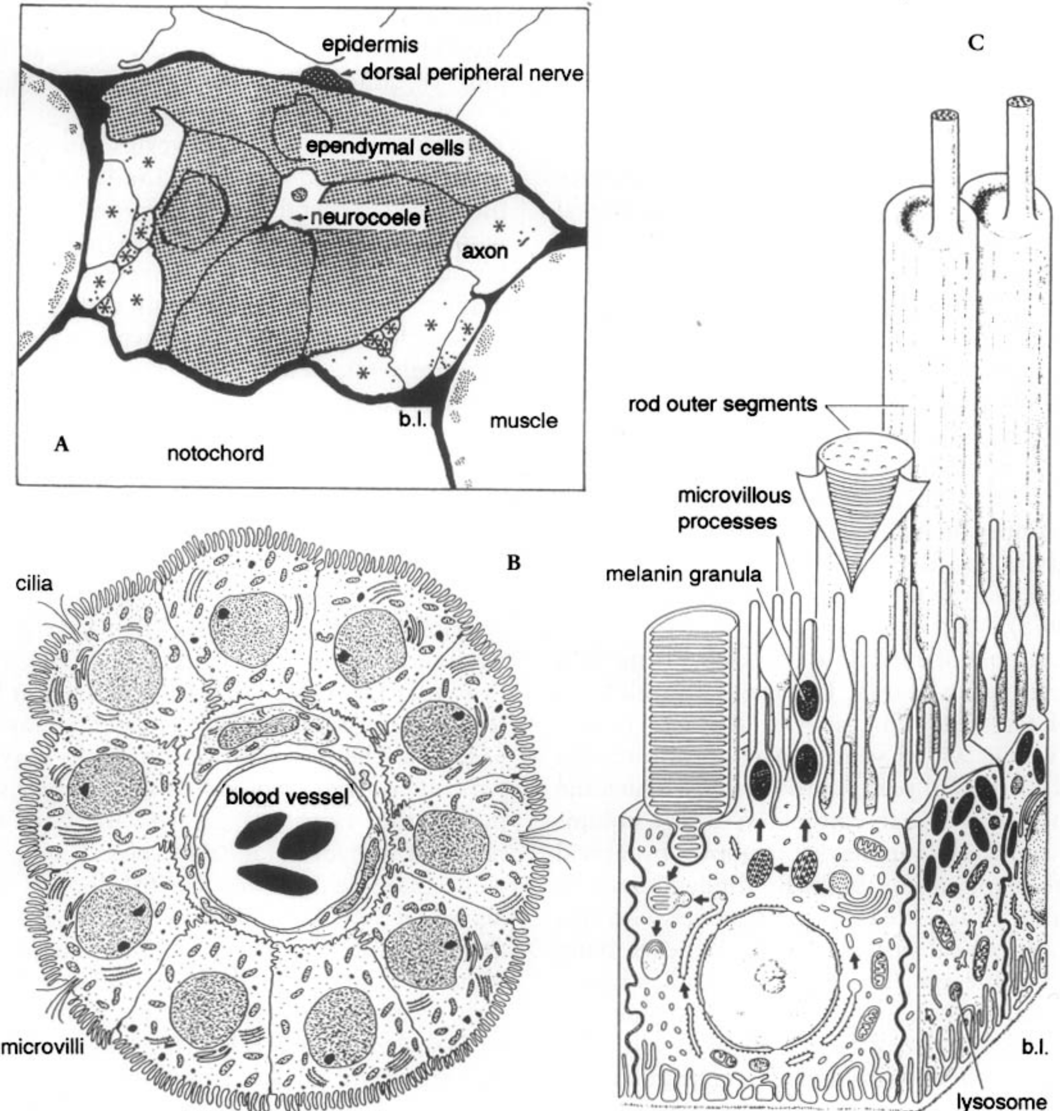


FIG. 4-5. "Epithelial" ependyma. (A) Transverse section through the central nervous system in the proximal tail region of a tunicate larva. The four ependymal cells are labeled by stippling, the seven axons at each side by asterisks. In the neurocoele, a cilium is visible [From Crowther and Whittaker (1992), with permission.] (B) Mammalian choroidal villus in cross section. The cuboidal epithelial cells have numerous microvilli on the apical membrane as well as tufts of cilia; lateral and basal membranes are infolded. A basal lamina (*not drawn*) lies beneath the epithelium. [From Dohrmann (1970), with permission. (C) Retinal pigment epithelium extending long (microvillous) processes between the outer segments of photoreceptor cells. [From Junqueira and Carneiro (1991), with permission.]

laris. Embryologically, the retinal pigment epithelium is formed from the medial wall of the optic vesicle, and its differentiation is probably induced by the neural retina. It functions as epithelium, macrophage, and glia (Steinberg, 1985).

The apical membranes of retinal pigment epithelium cells extrude profuse microvillous and sheath-like processes that are closely apposed to and ensheathe portions of the outer segments of rod and cone photoreceptor cells (Figure 4-5C, 4-12D). Apicolaterally, belts of tight junctions encircle the retinal pigment epithelium cells and contribute to the blood-retina barrier. The shed tips of photoreceptor outer segments (there is continuous renewal of outer segments by "disk shedding") are surrounded by pseudopodia from the apical retinal pigment epithelium surface and taken into the cytoplasm (Bok and Young, 1979). Such phagosomes are then fused with lysosomes and degraded. The cytoplasm is densely packed with rough endoplasmic reticulum and Golgi's complexes. These organelles are involved in melanin synthesis and the formation of the pigment granules ("melanosomes") that fill the apical cytoplasm and villous processes, and serve to absorb

stray light. Retinal pigment epithelium cells absorb fluid from subretinal space (and thus help to prevent retinal detachment), most probably by apical bicarbonate absorption (see Steinberg, 1985). The basal cytoplasm is rich in mitochondria to provide the energy necessary to drive this process, and the surface area of these cells is greatly increased by the apical microvilli and by elaborate infoldings of the basal plasma membrane.

The retinal pigment epithelium is continuous with similar epithelial ependymocytes. At the periphery, the retinal pigment epithelium undergoes a transition of into the pigmented outer layer of the ciliary epithelium. The latter extends up to the marginal fold where it is continuous with the inner nonpigmented layer of the ciliary epthelium. At the ora serrata, this nonpigmented ciliary epithelium changes over to cuboid ependymocytes that may be related to Müller cells of the adjacent neural retina (Pei and Smelser, 1968).

A final example of epitheloid ependyma is the lens of the parietal eye in lizards. This eye is derived from the roof of the embryonic neural tube as a noninvaginated vesicle whose ventral part constitutes the retina, and the dorsal part forms the lens. Thus, the parietal lens consists of an epithelium of high columnar ependymocytes that may contribute, by apical secretion, to maintenance of the fluid-filled lumen of the eye (Eakin et al., 1961). In congruence with this hypothesis, the apical surface of the cells bears many microvilli, and the apical part of the cytoplasm contains abundant mitochondria and vesicles (Eakin et al., 1961); the cytoplasm is rich in glycogen after dark adaptation. The basal (light-accepting) part of the cytoplasm contains a large fibrous mass with a dense spherical body at its center; the basal membrane abuts a basal lamina.

Central Nervous System Radial Glia I: Tanycytes. The term *tanycyte* (from the Greek *ταννειν* = to stretch) was introduced by Horstmann (1954) to describe (in sharks) ependymoglial cells whose processes extended over large distances. This term is now commonly applied to such ependymoglial cells of the adult brain and spinal cord (Figure 4-6). Tanycytes have been described in acrania, agnatha, selachian fish, teleostian fish, amphibians, reptilians, and birds (Stensaas and Stensaas, 1968a, 1968b; reviewed by Scharrer et al., 1980).

In adult mammals, tanycytes are restricted to the wall of the diencephalic third ventricle (Figure 4-7A, B, C), the dorsal and ventral walls of the mesencephalic aquaeduct (Figure 4-7D), the floor of the fourth ventricle, and the ventral part of the spinal central canal (Figure 4-7E). Tanycyte-like cells have also been reported in the lateral ventricles of adult rats (Hirano and Zimmerman, 1967) and in the velum medullare of adult primates (Reichenbach, 1990).

The various types of tanycytes can be classified in different ways. Horstmann (1954), for example, distinguished "ependymal tanycytes" (unipolar cells with their somata within the ependymal layer) from bipolar "extraependymal tanycytes" (with their somata elsewhere in the nervous tissue). Another discrimination can be made between (relatively short) "protoplasmic tanycytes" that have many side branches and protrusions within gray matter (e.g., Figure 4-7C, E), and (rather long) "fibrous tanycytes," whose processes are mainly smooth-surfaced, and extend through white matter (Figure 4-7A), although in many cases the cells have a shorter "protoplasmic" neck region and a longer "fibrous" tail process (Figures 4-7A, B). Finally, one may discriminate "pial tanycytes" that have processes extending to the pia mater (Figure 4-7A), from short "vascular tanycytes" that form endfeet at blood vessels in the proximity of the ependymal layer (Figures 4-7B, E). The processes of some cells may make *en-passant*-contacts to blood vessels on their way to farther distant targets (Figure 4-7B). With the exceptions of tanycytes in the ventral margin of the diencephalic third ventricle (Millhouse, 1971; Akmayev et al., 1973; Seress, 1980) and the velum medullare (Reichenbach, 1990), all mammalian tanycytes are of the vascular variety. These may be born rather late in ontogenesis (e.g., Altman and Bayer, 1978) whereas pial tanycytes may directly arise by differentiation from embryonic radial glia (see below).

Tanycytes have either one or no cilium, and various numbers of microvilli and larger protrusions from their apical surface (see Figure 4-3F). Together with endo- and/or exocytotic membrane vesicles, these specializations indicate an active exchange of substances with the cerebrospinal fluid. Neighboring tanycytes are interconnected via apicolateral junctions. In the hypothalamus as well as most circumventricular organs, adjacent tanycytes are interconnected by tight junctions that serve to separate the cerebrospinal fluid from the "blood milieu" surrounding the fenestrated capillaries (Brightman and Reese, 1969; cf. Leonhardt, 1980). The basal processes of all tanycytes contain abundant microtubuli and intermediate filaments, and extend variable numbers of lateral protrusions. These processes terminate in conical endfeet whose basal membrane contains orthogonal arrays of particles (Hatton and Ellisman, 1982).

Hypothalamic tanycytes have been studied exten-

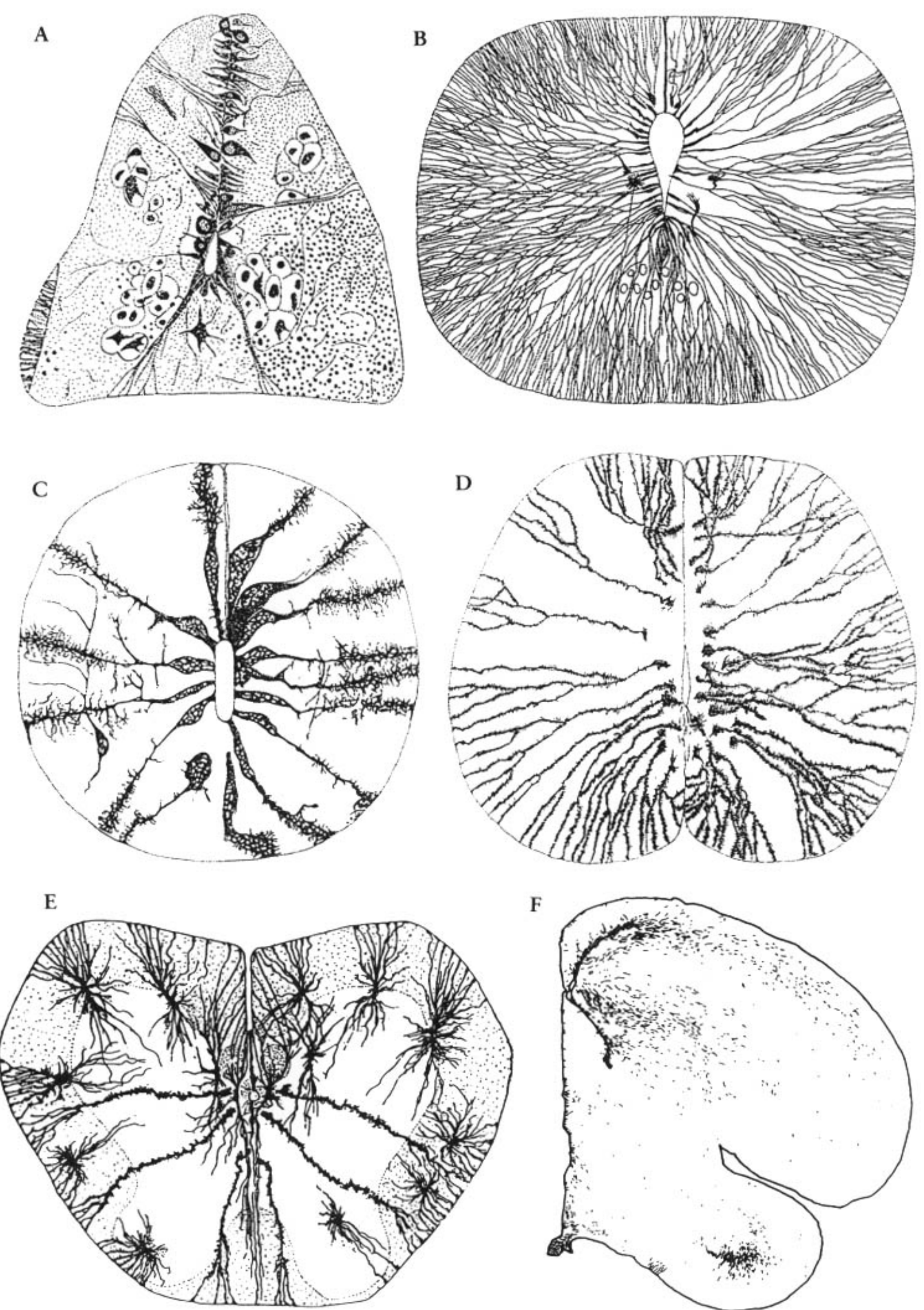


FIG. 4-6. Phylogenetic samples of radial glial cells in adult/subadult acrania and chordata. (**A**) Cross section of spinal cord of *Amphioxus* (acrania), sublimate-fixation. (**B**) Cross section of medulla oblongata of *Petromyzon* (lamprey; agnatha), Golgi impregnation. (**C**) Cross section of spinal cord of a young salmon of 160 mm length (teleostian fish; vertebrata), Golgi impregnation; (**D**) Cross section of spinal cord of a young frog (anuran amphibia, vertebrata), Golgi impregnation. (**E**) Cross section of spinal cord of a 20-day-old lizard (*Lacerta agilis*; reptilia, vertebrata), Golgi impregnation. (**F**) Frontal (half-) section of telencephalon of canary (*Serinus canaria*; aves, vertebrata), mapping of radial glia labeled with the anti-vimentin antibody 40E-C. [Figure A: from Rohde (1890); Figures B, C, D: from Retzium (1893); Figure E: from Ramón Y Cajal (1911); Figure F: from Alvarez-Buylla et al. (1988), with permission.]

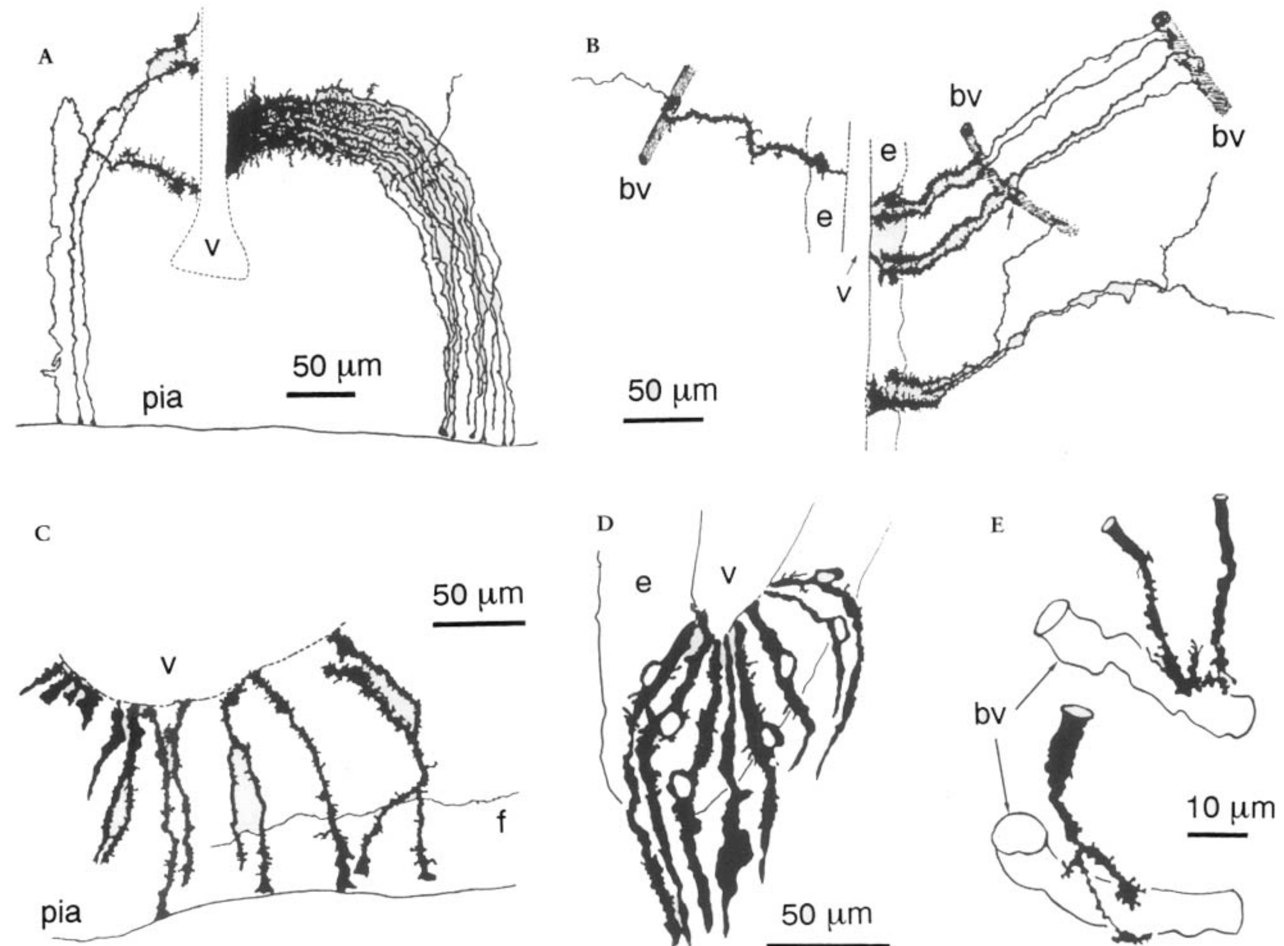


FIG. 4-7. Samples of radial glia in adult mammalian (rat) central nervous system, Golgi impregnation. (A) Ventral diencephalic tanycytes at the level of the anterior hypothalamus. (B) Diencephalic tanycytes at the dorsal level of the ventromedial hypothalamic nucleus. (C) Diencephalic tanycytes of the median eminence. (D) Mesencephalic tanycytes at the aqueduct (only the proximal parts of the cells are drawn). (E) Spinal tanycytes at the central canal (only the distal portions of the basal processes are shown). *e*, ependyma; *v*, ventricle; *bv*, blood vessel. [Figures A, B, C: from Millhouse (1971), with permission; Figure D: from Seress (1980), with permission; Figure E: from Rafols and Goshgarian (1985), with permission.]

sively, and on the basis of their morphology, topography, and metabolism, Akmayev and coworkers (1973) have distinguished several types of hypothalamic tanycytes. α1-Tanycytes (mostly vascular tanycytes) face the ventromedial nucleus (Figure 4-7B), and α2-tanycytes (long pial tanycytes) are found at the level of the arcuate nucleus (Figure 4-7A). The endfeet of β-tanycytes abut the blood vessel system of the pars tuberalis; β1-tanycytes are located in the lateral extensions of the infundibular recess, and β2-tanycytes line the floor of the ventricle (median eminence proper; Figure 4-7C). β-Tanycytes contain abundant lipid inclusions and smooth endoplasmic reticulum (e.g., Rodríguez et al., 1979), together with enzymes of lipid metabolism (Akmayev et al., 1973). The basal processes of β2- (but not β1-) tanycytes receive of up to 100 synapse-like contacts that arise partly from fibers of the tuberohypophyseal tract (e.g., Wittkowski, 1967). These contacts consist of vesicle-containing presynaptic terminals and an unspecialized postsynaptic membrane (Güldner and Wolff, 1973). The endfeet of infundibular tanycytes show varying organelle contents, probably related to their functional state (Rodríguez et al., 1979). These endfeet may occupy variable surface areas on the fenestrated blood vessel plexus, and, thus, regulate the available contact areas of neurohemal axons in dependence on functional requirements (Wittkowski, 1973; Lichtensteiger et al., 1978).

Tanycytes can be more than 20 mm long in the fetal primate brain (Rakic, 1984). Lengths of several millimeters have been reported in the adults of nonmammalian vertebrates (e.g., selachians: Horstmann, 1954; amphibians: Paul, 1967; reptilians: Stensaas and Stensaas, 1968b; birds: Alvarez-Buylla

et al., 1988). In adult mammals, fibrous tanycytes 2 mm long have been described in the floor of the fourth ventricle in rabbits (Felten et al., 1981). Fibrous tanycytes of more than 500 μm in length have been found within the velum medullare of monkeys (Reichenbach, 1990), and in the hypothalamic region of monkeys and rats (Seress, 1980). In contrast, protoplasmic tanycytes are never longer than about 250 μm (Millhouse, 1971; Lichtensteiger et al., 1978; Bruni et al., 1983). The length of these processes may be limited by their capacity to act as "cables" for spatial buffering potassium currents (Reichenbach et al., 1987).

Central Nervous System Radial Glia II: Tanycyte-like Cells of Circumventricular Organs. The ependymal ceiling of the ventricular system is not uniform. Characteristic ependymal specializations, "circumventricular organs," are located at, or near, the median axis of the central nervous system. Their distribution is a legacy of the embryology of the brain: whereas the lateral plates of the neural anlage generate many neurons and differentiate into large tissue blocks, parts of the roof plate, floor plate, and lamina terminalis undergo very restricted neurogenesis. These regions remain "ependymal" and form, in collaboration with blood vessels, peculiar ependymal "organs." Most of these organs lack a blood-brain barrier; in these cases, the ependymocytes are interconnected by apicolateral tight junctions (see also the preceding section *Central Nervous System Radial Glia I . . .*). Circumventricular organs are more common in "lower" vertebrates than in mammals. Space limitations prevent us from reviewing all of the organs here; the interested reader should consult Vigh (1971) for a catalogue of these organs. The choroid plexus is one of the circumventricular organs, and its ependymoglial cells have been already introduced (see the section *Epithelial Ependymocytes*). Details of ependymocytes from two further selected organs are discussed below.

The most intensely studied circumventricular organ is the subcommissural organ. It lies at the border of the diencephalon and mesencephalon and surrounds the dorsal entry of the cerebral aqueduct. The subcommisural organ is present in early development in all vertebrates, but by adulthood it is often substantially reduced or missing (e.g., in humans: Oksche, 1964). In adult heterothermic vertebrates and in fetal birds and mammals, these cells have a tanycyte-like shape; they lose their long basal processes in adult warm-blooded animals (Oksche, 1961). The ultrastructure of these cells indicates a very active secretory function: abundant mitochondria, elaborated endoplasmic reticulum, and secretory vacuoles are tightly packed in their cytoplasm (Figure 4-8A). The main secretory product is a particular glycoprotein, and is released into the ventricular lumen where it aggregates and forms Reissner's fiber. This aggregation is supported and oriented by the kinocilia of the ependymocytes, by the flow of the cerebrospinal fluid, and by stretching forces of the already formed fiber. The fully formed fiber can be up to 50 μm thick and more than 1 m long, since it extends down to the ventriculus terminalis of the spinal cord. A growth rate of several millimeters per day has been observed in most species studied (e.g., Ermisch et al., 1971). Reissner's fiber is also found in *Amphioxus* and in some tunicate larvae, which indicates that this enigmatic strand predates the evolution of the vertebrate nervous system (Bone, 1981). The function of Reissner's fiber is unknown, but there is some evidence that it may be involved in helping to organize the axis of the developing body (Rühle, 1971).

Another circumventricular organ is the saccus vasculosus, which occurs only in fish, as an outgrowth of the hypothalamic floor. The ependyma of this organ consists of coronet cells and "supporting cells" (i.e., microvilli-rich tanycytes), while the neuronal element are cerebrospinal fluid-contact neurons ("pear-shaped cells" in Figure 4-8B). The ependymal cells are interconnected by tight junctions, desmosomes, and zonulae adhaerentes (Jansen and Flight, 1969). Their basal processes form endfeet at the basal lamina that separates the ependyma from the underlying mesenchyme. A complex arrangement of blood vessels, consisting of capillaries and sinuses lies beneath the mesenchymal sheath (Figure 4-8B). The coronet cells owe their name to their apical (flowerlike) protrusions (the "coronets") that consist of up to 20 end-bulbs of modified $9 \times 2 + 0$ cilia. These end-bulbs increase the apical surface area about 800-fold (Jansen, 1975), and seem to consist of convoluted tubules with a diameter of 50 to 150 nm. The apical cytoplasm of these cells is tightly packed with smooth endoplasmic reticulum. The basal cytoplasm contains many mitochondria and various types of granules. Coronet cells are targets of synaptoid contacts (Jansen and Flight, 1969). It has been suggested that coronet cells have a secretory function, and possibly assist osmoregulation (e.g., von Mecklenburg, 1973), by cation transport (Jansen, 1975).

Central Nervous System Radial Glia III: Retinal Müller Cells. Insofar as the retinas of both lateral and pineal/parietal eyes are embryonic outgrowths of the diencephalon, their radial glial cells belong to the diencephalic tanycyte group; on the other hand, eyes

may be considered to be circumventricular organs (Leonhardt, 1980). The radial fibers of lateral eye glia were discovered by H. Müller in 1851, and the cells now bear his name. They appear to be present in the retinas of all vertebrates, including those of agnatha (Rubinson, 1990).

Similar cells have been described in the parietal/frontal eyes of lizards and frogs (Eakin, 1973; Engbretson and Linser, 1991) (see Figures 4-8C, and 4-9A) but not in birds and mammals, where the pineal is differentiated as an endocrine gland and does not show the structure of a sensory epithelium. In reptilian parietal eyes, the apical border of the supporting cells bears microvilli and cilia that project into the lumen of the organ. Their apical cytoplasm contains numerous mitochondria, filaments, vesicles, vacuoles, lipoid bodies, and a Golgi complex. The cells form tight and adherent junctions with adjacent cells. The cells are bipolar, and extend a basal process that terminates in a flattened endfoot at the basal lamina covering the underlying mesenchymal tissue. Like retinal pigment epithelium cells, the supportive cells of the reptilian parietal eye contain pigment granules that are probably composed of melanin (Eakin, 1973; Engbretson and Linser, 1991). On the other hand, they bear biochemical similarities to Müller cells of the lateral eyes.

Müller cells are bipolar tanycytes whose somata lie within the inner nuclear layer (where they may form a distinct median sublayer in some species). They constitute the principal glia of the retina and span its entire thickness. Although Müller cells from different species vary considerably in shape (Figure 4-9B–O), some features are fairly universal. At the level of the outer limiting membrane, Müller cells extend apical microvilli into the subretinal space, between the inner segments of photoreceptor cells (Figures 4-3E, 4-9, 4-10). In some species, Müller cells also possess a cilium (Ennis and Kunz, 1986). The length and number of microvilli vary between species, probably in inverse relation to the degree of vascularization (Uga and Smelser, 1973; see, however, Dreher et al., 1992). Apicolaterally, Müller cells are connected to their neighboring Müller and photoreceptor cells by specialized junctions to form the outer limiting "membrane." In most vertebrate species studied these junctions are zonulae adhaerentes or (in fish) tight junctions. However, amphibian Müller cells are connected instead by intermediate junctions, and (in frogs and toads) by gap junctions (Uga and Smelser, 1973) that permit extensive electrical coupling (Attwell et al., 1985). Although human Müller cells are reported to express gap junctions in the inner retina (Reale et al., 1978), neither

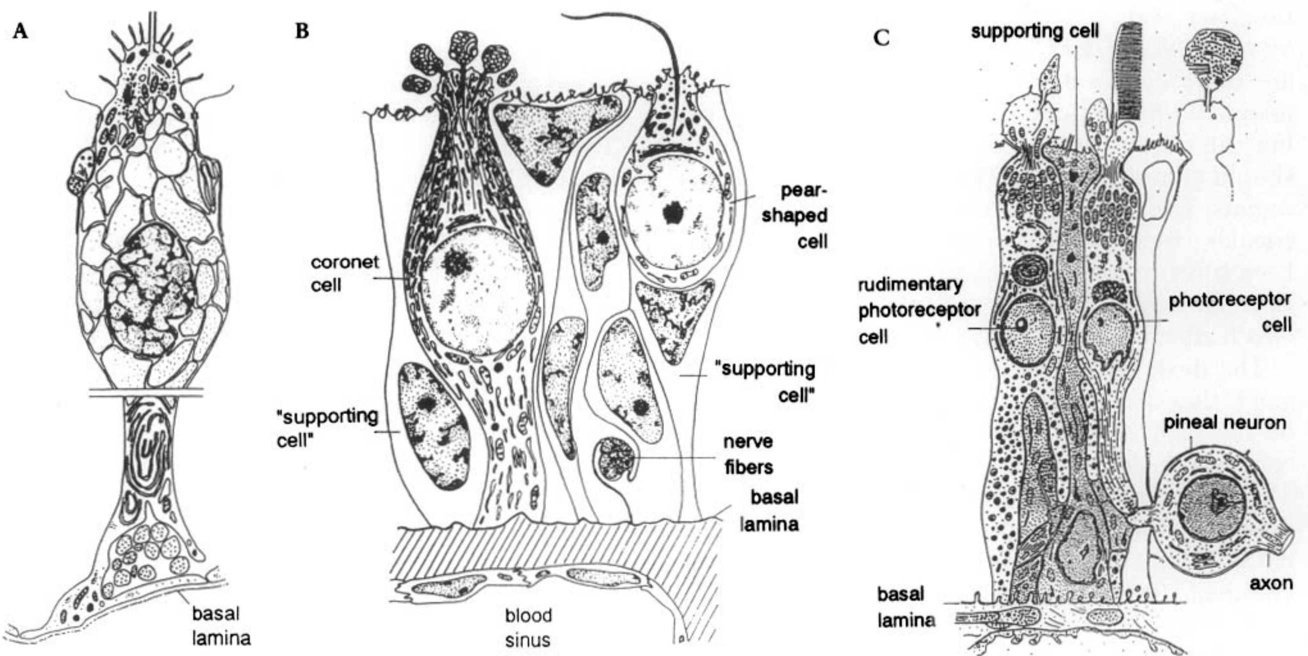


FIG. 4-8. Specialized ependymal cells in the "midline organs." (A) Cell of the subcommissural organ of *Bombina variegata* (toad, anuran amphibia). [From Altner (1968), with permission]. (B) Epithelium of the saccus vasculosus of *Spinax niger* (selachian fish), containing coronet cells, pear-shaped cells, and "supporting" ependymal cells. [From Graf von Harrach (1970) with permission.] (C) Parietal organ of the adult wall lizard (*Lacerta muralis*), illustrating the sensory epithelium. [From Collin and Oksche (1981), with permission.]

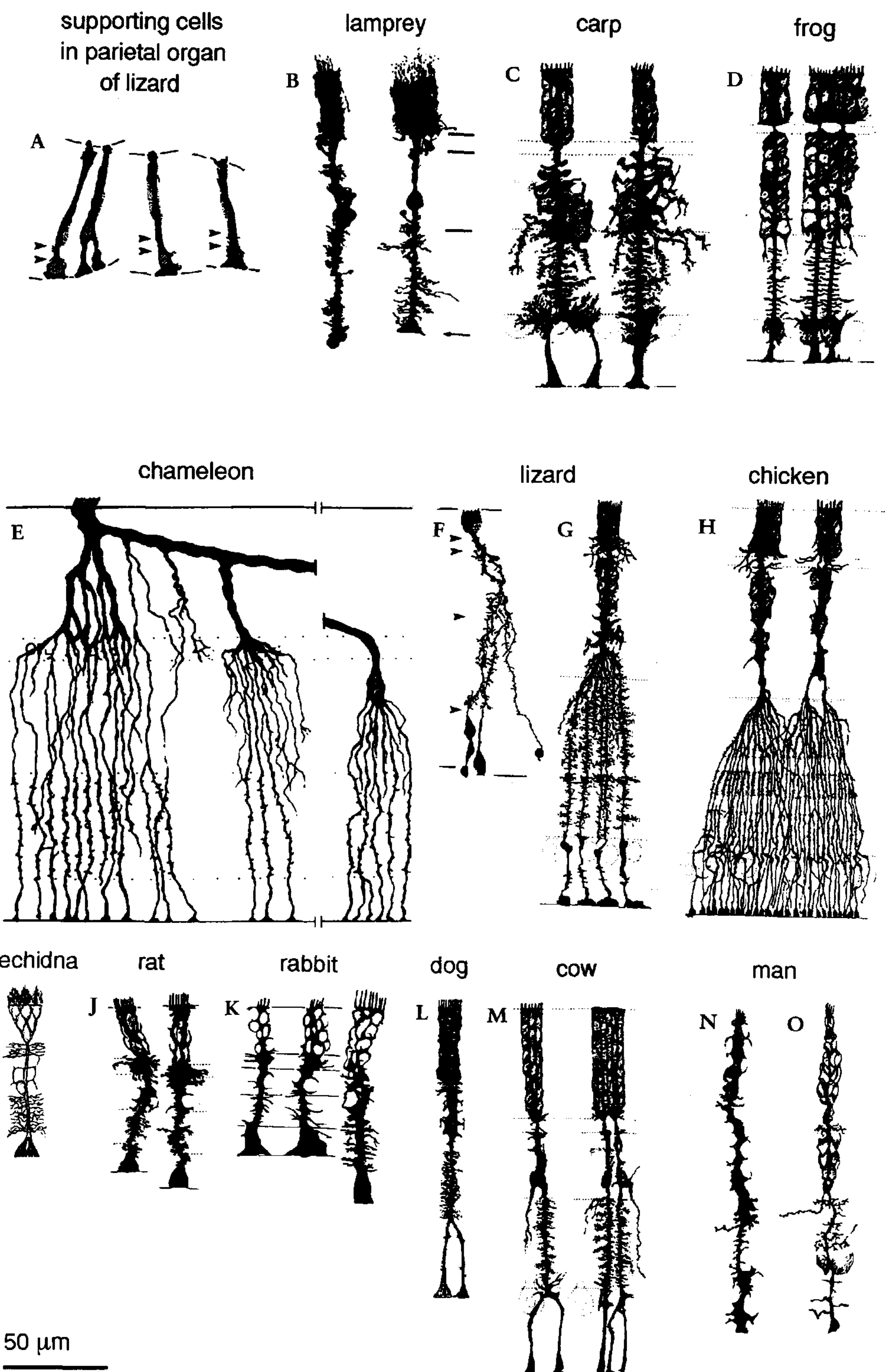


FIG. 4-9. Retinal radial glial cells. Shown are supporting cells of lizard parietal organ (*top left*) and Müller cells of various vertebrate species. Golgi impregnation, with the exception of the *Echidna* Müller cell that is a camera-lucida drawing of an immunocytochemically stained specimen. Note that the chameleon Müller cell is only partly shown; the transversal process is interrupted for a length of several hundred micrometers. At the top is the apical process of the cells (reaching the subretinal space), at the bottom are the vitread endfeet. The calibrating bar gives only an approximate idea of different cell sizes, since Ramón y Cajal's and Dogiels' drawings are without calibration. [Figures A, F: from Engbretson and Linser (1991), with permission; Figure B: Rubinson (1990), with permission; Figures C, D, G, H, L, M, N: from Ramón y Cajal (1892); Figure O: Dogiel (1893); with permission; Figure E courtesy of F. A. Prada; Figures I, J, K: our own unpublished data.]

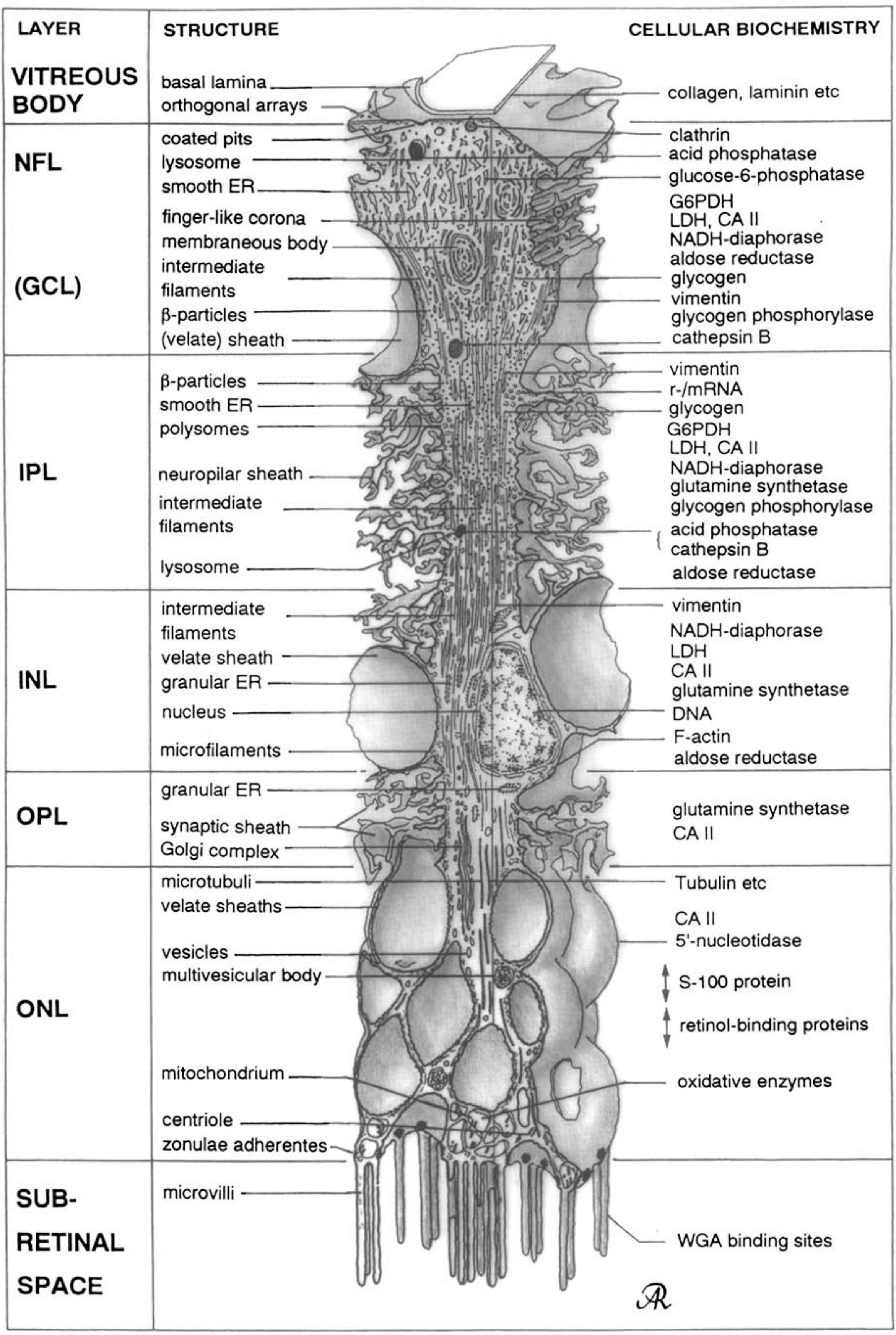


FIG. 4-10. Survey of the cytotopographica specializations of a rabbit Müller cell. *NFL*, nerve fiber layer; *GCL*, ganglion cell layer; *IPL*, inner plexiform layer; *INL*, inner nuclear layer; *OPL*, outer plexiform layer; *ONL*, outer nuclear layer. [Slightly modified from Reichenbach (1989), with permission.]

gap nor tight junctions have been unequivocally demonstrated in the apicolateral membrane of mammalian Müller cells *in situ*. Gap junctions between Müller cells and astrocytes have been demonstrated in the rabbit retina with dye-coupling (Robinson, 1992: Robinson et al., 1992), but Müller cells are never dye-coupled to each other.

Müller cells send side branches into the two plexiform (synaptic) layers, where they form sheaths around neuronal processes and synapses, particularly around the photoreceptor pedicles in the outer plexiform layer (Reichenbach et al., 1989) (see Figures 4-3E, 4-9, and 4-10). They wrap most, but not all, neuronal profiles in the plexiform layers. In the nuclear layers, the lamellar processes of Müller cells form basketlike structures that envelop the cell bodies of neuronal cells (Ramón y Cajal, 1892; Hama et al., 1978; Reichenbach et al., 1988, 1989; Dreher et al., 1992) (Figures 4-9 and 4-10). Müller cells extend smooth and sometimes rather long processes through the nerve fiber layer. Like astrocytes at the nodes of Ranvier, Müller cells send side branches that form finger-like coronae of fine processes abutting node-like specializations of nerve fibers (Hildebrand and Waxman, 1984; Reichenbach et al., 1988).

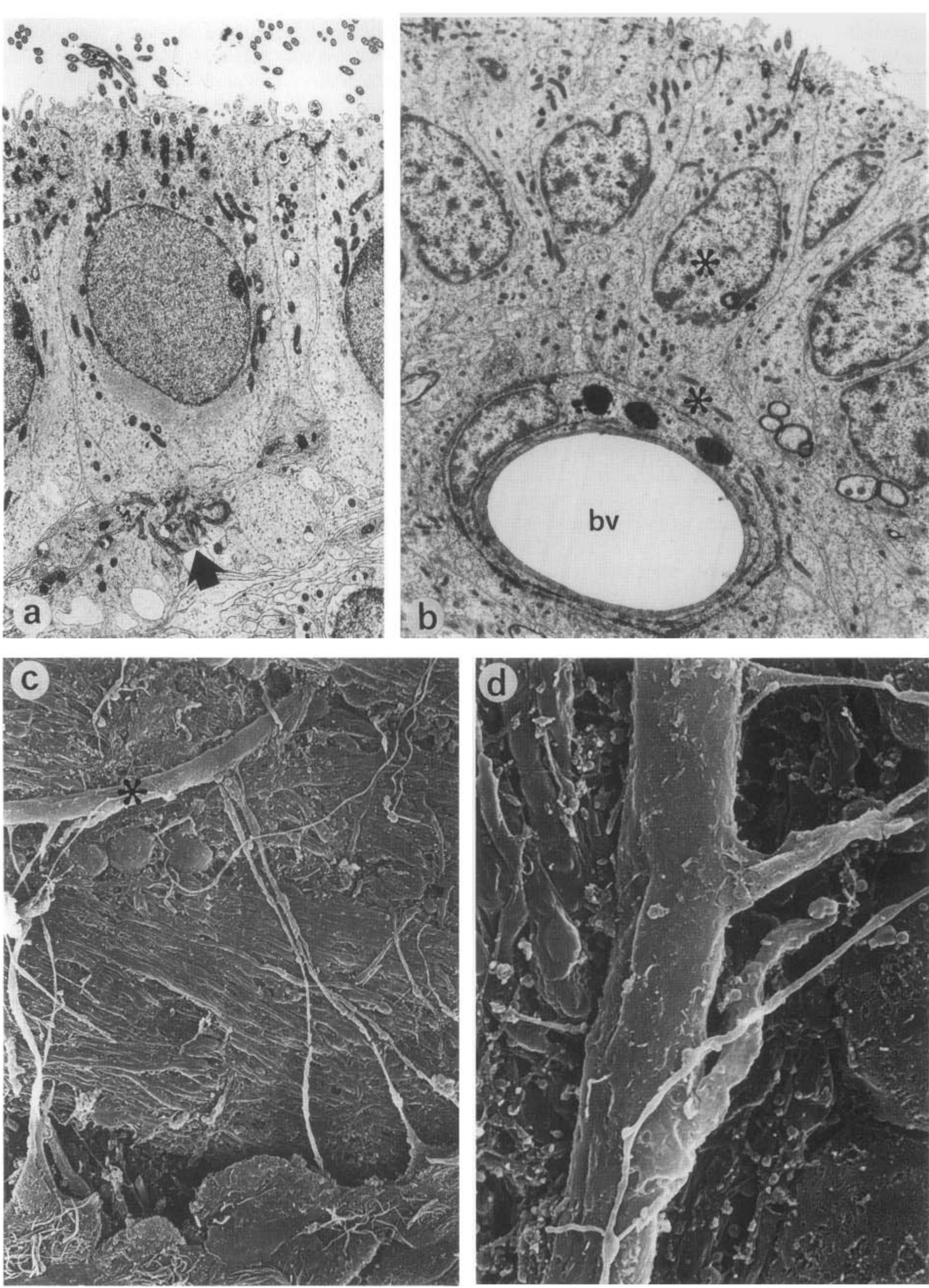
The basal end of each Müller cell trunk terminates in a funnel-shaped endfoot, which lies adjacent to the inner limiting membrane. This membrane is a basal lamina that is at least partly produced by Müller cells. Müller cell endfoot plates form a mosaic that can be revealed by silver impregnation (Wolter, 1961), scanning electron microscopy (Masutani-Noda and Yamada, 1983), immunocytochemistry (Dreher et al., 1988), and freeze-fracture electron microscopy (Wolburg and Berg, 1988; Richter et al., 1990). Each endfoot extends some side branches that overlap with similar branches from neighboring endfeet. The vitread endfoot membranes of all vertebrates except anurans express orthogonal arrays of membrane particles (Reale et al., 1974; Wolburg and Berg, 1988; Richter et al., 1990; Wolburg et al., 1992). In species with vascular retinas, the same membrane specializations are found where Müller cells form *en-passant* endfeet onto retinal blood vessels (Wolburg and Berg, 1988). Some Müller cells have been observed that did not reach the inner limiting membrane but terminated at a large intraretinal blood vessel (Reichenbach et al., 1992a).

As a rule, Müller cells in avascular retinas have mitochondria only in the scleradmost portion of their cytoplasm (see, e.g., rabbit: Figure 4-10), whereas in retinas with intra- or epiretinal blood vessels, mitochondria are also found in their vitread endfeet (Uga and Smelser, 1973). Amphibian Müller cells are exceptional in several respects (see above). They express lateral membrane infoldings near both outer and inner limiting membranes (Uga and Smelser 1973) (see also Figure 4-3E), and large extracellular spaces occur around their endfeet, (Lasansky and Wald, 1962). Reptilian and avian Müller cells are conspicuous by the elaborate subdivision of their vitread process into many branches where the trunk enters the inner plexiform layer (Figure 4-9). In chameleons, Müller cell trunks follow a tangential course near the fovea, but these cells also give rise to supplementary trunks that course radially toward the inner limiting membrane (Ramón y Cajal, 1911; Prada et al., 1979) (Figure 4-9E). There are often striking quantitative and qualitative differences between Müller cells that vary systematically with eccentricity across a single retina (Dogiel, 1893; Prada et al., 1979; Prada et al., 1989; Reichenbach and Wohlrab, 1986; Reichenbach et al., 1988; Reichenbach et al., 1989; Robinson and Dreher, 1990; Dreher et al., 1992) (see also Figure 4-14).

Ependyma *s. str.*; Ciliated Ependymocytes. In adult mammals, the ventricular lining is mainly composed of cuboid ciliated ependymocytes. These cells may be columnar with a height of up to 15 μm or as flat as 0.1 μm, depending on location; they tend to be taller over gray matter than white matter. In hydrocephalus they become very flattened (Weller et al., 1971).

The apical surface of an ependymocyte is characterized by variable numbers of microvilli and protrusions, and 12 to 60 kinocilia, which vary in number according to the species (Brightman and Palay, 1963) (Figures 4-11 and 4-12). The cilia are 10 to 20 μm long, and are of the $9 \times 2 + 2$ type. These cilia beat rhythmically at a frequency of about 200 oscillations per minute (Singer and Goodman, 1966) and appear to assist the rostrocaudad flow of cerebrospinal fluid.

Ependymocytes are connected by apicolateral zonulae adhaerentes and gap junctions. The fact that ciliated ependymocytes lack tight junctions permits free exchange between the extracellular space of the brain and the cerebrospinal fluid (e.g., Brightman and Reese, 1969). Ependymocytes have a cytoplasm that is rich in intermediate filaments and large mitochondria. Their basal surface terminates directly onto the basal lamina of a blood vessel (Figure 4-11B), or onto a "basement membrane labyrinth" (Figure 4-11A). Such labyrinths are the remnants of an embryonic vascular network in the subventricular zone (Leonhardt, 1970; Booz et al., 1974). Fine basal processes of ciliated ependymocytes may extend over considerable distances to meet the basal lamina of sube-


a
b
bv
c
d

pendymal blood vessels (Hirano and Zimmerman, 1967); in contrast to those of tanycytes, these basal processes are oriented transversely rather than orthogonally to the ependymal surface (Inokuchi et al., 1988) (Figure 4-11C, D). Rodríguez et al. (1985) state that every ependymocyte contacts a basal lamina at its basal pole.

GENERAL FEATURES OF EPENDYMOGLIAL CELL PROCESSES

Types of Ependymal Cell Processes as Determined by Contacts to Different Microenvironmental Compartments

When the structure and ultrastructure of various ependymal cell types is compared, a series of striking similarities becomes apparent. It has been suggested by Reichenbach (1989) that contact to similar types of microenvironment may induce similar features of the contacting cell processes. Figure 4-13 gives a survey on the diversity of types of processes that, in principle, may occur on ependymoglial cells; for a review of functional specializations of these processes, see Reichenbach (1989).

Type I: The Apical Pole of Neural Epithelium. By definition, a process of type I (i.e., a process contributing to the apical pole of the neuroepithelium) is an obligatory feature of every ependymoglial or ependymoglia-like cell. Usually, its tip faces a fluid lumen or space, into which it extends many microvilli. The apical pole contains abundant mitochondria, features that indicate high metabolic activity, which is presumably related to active exchange of substances with the luminal fluid. The supporting cells of the inner ear (whose apical pole is confronted to endolymph resembling the intracellular milieu in many respects) seem to be an exception to this pattern, since apical microvilli and mitochondria are rare if not absent (Figure 4-3D). A further characteristic of apical processes in some but not all ependymoglia are kinocilia of the 9 × 2 + 2 type. These kinocilia seem to be phylogenetically conservative property, since they are present in ependymoglia-like cells of primitive deuterostomian nervous systems (Figure 4-3A, and 4-5A), as well as in cells of the "ciliary bands" of deuterostomian larvae (Figure 4-2) that may resemble the evolutionary precursors of the vertebrate neural tube (e.g., Lacalli et al., 1990; Crowther and Whittaker, 1992).

Finally, type I processes are interconnected by various types of apicolateral junctions. These (in particular, desmosomes) are general "markers" of virtually all epithelial cells and occur very early in development (Revel and Brown, 1976). Thus, their expression may be genetically determined. On the other hand, their nature varies considerably depending on the local microenvironment. In regions where no endothelial blood-brain barrier exists (e.g., most circumventricular organs; retinal pigment epithelium), but not elsewhere, ependymoglial cells form a cerebrospinal fluid-brain barrier by the expression of tight junctions (cf. the situation in elasmobranchs; see Chapter 6). Apicolateral gap junctions occur between Müller cells in frogs but not in mammals; when, however, rabbit Müller cells form homogeneous cultures *in vitro*, gap and even tight junctions can be observed (Wolburg et al., 1990).

There are further examples of modification of process-specific features. When the basal pole of retinal glial (Müller) cells is directly exposed to the vitreous humor (after basal lamina defects in retinal wounds) the cells send microvilli into the lumen of the eye (Foos and Gloor, 1975; Miller et al., 1986). Retinal pigment epithelium cells proliferating under areas of retinal detachment may lose their basal lamina contact and face the subretinal fluid round about; in this case, their entire surface is covered by microvilli (Anderson et al., 1983).

Thus, the microenvironment seems to determine not only the occurrence but also many of the particular features of type I processes.

Type II: The Basal Mesenchymal Contact. Whereas the contact with the apical surface of the neural epithelium is a primary feature of neuroectodermal cells that is retained even during mitotic cell proliferation (which principally occurs at the ventricular surface) (Sauer, 1935), the basal pole of the epithelium can be reached only by the growth of basal processes. From cell culture experiments, there is some evi-

FIG. 4-11. Mammalian ependymal cells. (**A**) Transmission electron microphotographs of rat third ventricular ependyma. × 6000. The basal pole of the ependymal cell abuts a basal membrane labyrinth (*arrow*). (**B**) Transmission electron microphotograph of rabbit lumbar ependyma. ×6000. The basal pole of at least one cell (*asterisks*) is shown to abut a blood vessel (*bv*). (**C, D**) Scanning electron microphotographs of the wall of the rat third ventricle near the median eminence. ×5000. The subependymal zone is partially exposed by application of collagenase and trypsin. Flattened ependymal cells with scanty cilia (bottom part of Figure C) extend long basal processes that attach to a capillary (*asterisk*); Figure D is a detail of Figure C and shows the endfeet at the capillary. ×25,000. [Figures A and B were kindly provided by Prof. H. Leonhardt, Kiel. Figures C and D: from Inokuchi et al. (1988), with permission.]

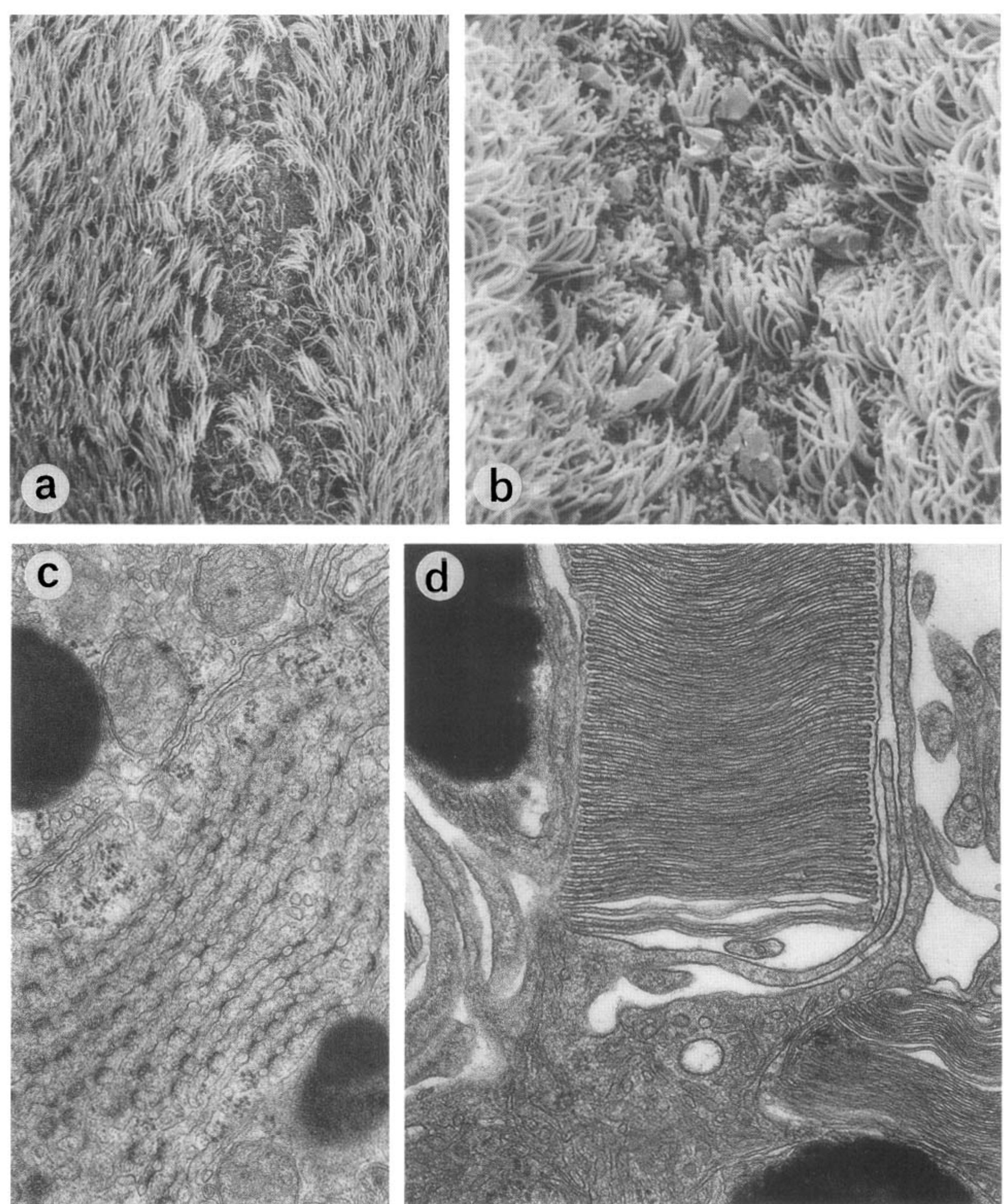


FIG. 4-12. (A, B) Scanning electron microphotographs of apical surfaces of ependymal cells in the ventral wall of the spinal central canal. (A) Line of cilium-poor ependyma within the usual cilium-rich ependyma; rat spinal cord. ×612. (B) The same region in rabbit spinal cord. ×2160. (C, D) Transmission electron microphotographs of human retinal pigment epithelium. (C) Annulate lamellae. × 32,000. (D) Enveloping sheaths around a photoreceptor outer segment (upper median part), pigment granulum within a microvillum (left upper part), and ingested packet of discs (right lower part). ×40,000. [Figures A and B were kindly provided by Prof. H. Leonhard, Kiel, and Figures C and D by Dr. I. Wood, San Francisco.]

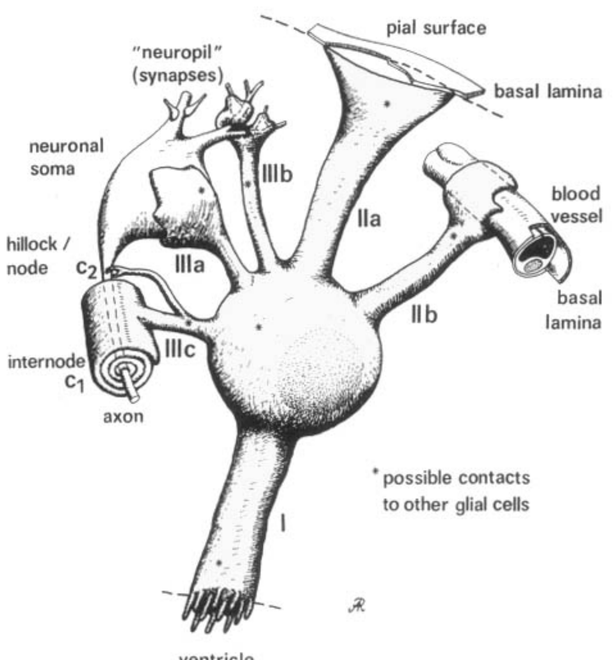


FIG. 4-13. "Idealized" ependymoglial cell expressing all types of contacts to microenvironmental compartments that such a cell can have. The three types of processes are (*I*) abutting the ventricular surface, (*II*) abutting a basal lamina, either of the pia mater (*IIa*) or of a blood vessel (*IIb*), and (*III*) ensheathing neuronal compartments as somata (*IIIa*), synaptic neuropil (*IIIb*), or axons (*IIIc*). [From Reichenbach (1989), with permission.]

dence of an inherent tendency toward bipolar growth by ependymoglia (e.g., Hatten, 1984) that may be stimulated by neighboring neurons (Hatten, 1985); on the other hand, mesenchymal cells seem to exert a chemoattractive influence on ependymoglial cell processes (D. Hartmann, personal communication, Kiel, 1994). In any case, the basal processes of ependymoglia form endfeet onto the basal lamina of the mesenchymal layer(s) underlying the nervous epithelium (type IIa), or the basal lamina of blood vessels (type IIb) (see Figure 4-13). The latter occurs more frequently at later stages of development when the tissue becomes thicker and vascularized.

Endfoot (footplate) membranes are connected to the basal lamina by hemidesmosomes, and express orthogonal arrays of intramembrane particles (Reale et al., 1974; Hatton and Ellisman, 1982; see also Chapter 41, this volume). The occurrence of vesicles concerned with endo-, exo-, or pinocytosis and coated pits indicates active material exchange with the compartment behind the mesenchymal basal lamina (i.e., blood plasma, vitreous body, subarachnoidal fluid, or perilymph). Ependymoglial type II processes are characterized by a cytoskeleton that is reinforced with abundant intermediate filaments. These filaments consist primarily of vimentin when the endfoot is in contact with cerebrospinal fluid or vitreous humor but of glial fibrillary protein when a blood vessel is contacted (Pixley and De Vellis, 1984; see also Chapter 43, this volume). Supporting cells of invertebrates (Bargmann et al., 1962) as well as those in the vertebrate peripheral nervous system (Angelborg and Engström, 1972) may contain very particular filaments.

When, under pathological or experimental conditions, apicolateral membranes of retinal pigment epithelium cells (Korte et al., 1986), Müller cells (Korte et al., 1992), or ependymocytes (Rosenstein and Brightman, 1979) are confronted with mesenchyma, they lose their original features and develop an endfootlike structure. This response suggests that mesenchymal contact (collagen?, laminin?) inhibits cell proliferation and process growth, and stimulates filament formation (Goetschy et al., 1987) and the production of basal lamina (Crawford, 1983; von Knebel Doeberitz et al., 1986). Thus, as was discussed for type I processes, the microenvironment seems to determine not only the occurrence but also much of the particular features of type II processes.

Type III: The Contact with Neurons. Type III processes are characterized by the formation of flat or lamellar sheaths that enclose neuronal somata (IIIa), synapses (IIIb), or axonal internodes ($IIIc_1$), or by fingerlike extensions (Carlstedt, 1977; Waxman, 1983) that contact the nodal specializations of axons ($IIIc_2$) (see Figure 4-13). These processes may act as stores of sodium or calcium ions (e.g., Gambetti et al., 1975).

In contrast to type I and type II contacts, which may establish early in ontogeny, specialized type III processes cannot be elaborated until neurons have completed their differentiation (Waxman et al., 1983). Indeed, the formation of such processes may be triggered by the onset of neuronal activity (Reichenbach and Reichelt, 1986), and their growth may be attracted (or repelled) by signals from active neurons. Potassium ions (Reichenbach and Reichelt, 1986; Reichelt et al., 1989) and neurotransmitters, such as glutamate (Cornell-Bell et al., 1990) and γ-aminobutyric acid (GABA) (Kettenmann et al., 1991), have been demonstrated or suggested as such neuronal signals. Several second messenger systems seem to be involved. Protein kinase C accelerates protein synthesis-dependent growth of long, branching processes (Althaus et al., 1991). In contrast, protein kinase A induces growth of short processes which extend flat membraneous sheaths from their growth cones; this might be important for ensheathing of neuronal elements (Althaus et al., 1990). A survey on the cascade(s) of signal trans-

mission has been provided by Arenander et al. (1988).

As was the case for type I and II processes, the microenvironment seems to determine not only the occurrence but also much of the particular features of type III processes.

Shape and Shaping of Cell Processes

It is long known that fibrous glia, which are found mainly in the white matter, can be distinguished from protoplasmic glia, which reside mostly in the gray matter. More recently, "velate astrocytes" were recognized by Chan-Palay and Palay (1972). These cells give rise to "bubble"- or basket-like sheaths around somata of small neurons. It has been pointed out that if it is long enough, the character of a single glial cell process can change from "protoplasmic" to "fibrous" as it passes from gray to white matter (Millhouse, 1971; Miller and Liuzzi, 1986) (Figure 4-7A). Müller cells may express up to three different morphologies as their processes pass through the various retinal layers. This variation is particularly evident in the rabbit retina, which contains true "white matter" in the form of a myelinated nerve fiber layer (Magelhães and Coimbra, 1972; Reichenbach et al., 1989; Figures 4-3E, 4-9, 4-10, and 4-14). Thus, within the outer and inner nuclear layers, Müller cell processes (and somata) are of the "velate" type, whereas in the plexiform (synaptic) layers they extend many fine side branches, and are "protoplasmic" in nature. Finally, within the nerve fiber layer Müller cell processes are smooth and "fibrous." Thus, glial cell processes seem to be shaped to a

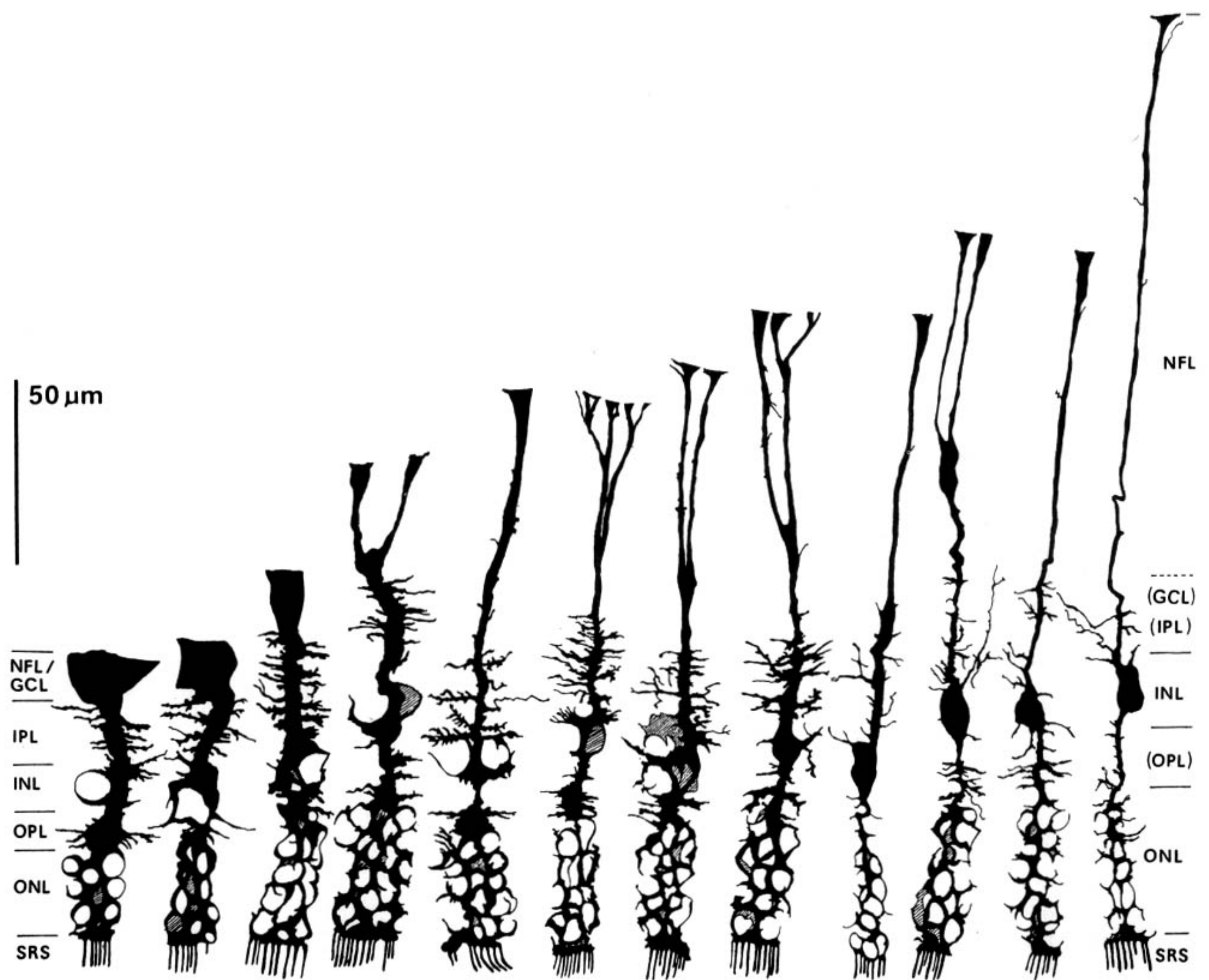


FIG. 4-14. Golgi-impregnated Müller cells from various regions of the rabbit retina (*left side*); far periphery (*right side*); medullary rays (*center*). *SRS*, subretinal space; see caption of Figure 4-10 for other symbols. [From Reichenbach et al. (1989), with permission.]

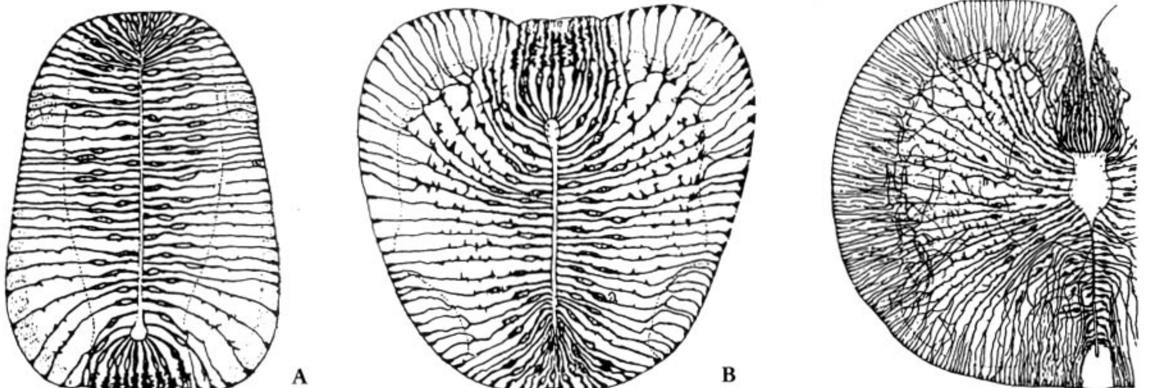


FIG. 4-15. Cross sections through the embryonic spinal cord of (A) chicken (3rd day of incubation = *E3*), (B) chicken (*E5*), and (C) human embryo, 44 mm long. Camera-lucida drawings of Golgi-impregnated neuroepithelial-radial glial cells. Note that the figures are not shown at equal magnification [From Ramón y Cajal (1911).]

large extent by their local (neuronal) environment, as if the glial side branches had been grown into the available interspaces between established neuronal compartments.

This principle is nicely illustrated by the fact that radial glia born after a marginal zone (i.e., future white matter) has formed, show characteristic splitting of their trunks as they enter this zone (Figures 4-6, 4-9, 4-14, and 4-15). This pattern suggests that axon bundles resist the ingrowth of glial processes, and that the growing tips of radial glia respond by issuing several sprouts that try to find a way around these obstacles. Similarly, in reptilian and avian retinas, where a thick inner plexiform layer is elaborated early in ontogeny, the Müller cell trunks split into numerous trunklets as they enter the inner plexiform layer (Figures 4-9F, H). Furthermore, if the viscosity of the medium is increased by addition of agarose (0.1 to 1.5%), the process growth of cultured glial cells is greatly impaired (H. Althaus, personal communication, Göttingen, 1993). While the preceding observations suggest that (at least, growing) glial cell processes are pliable compared with those of neurons, specialized ependymoglia-like cells (e.g., in Corti's organ) seem to provide mechanical support to neurons.

The shape of cell processes greatly influences their surface:volume ratio, which can be translated as membrane:cytoplasm ratio, and which can be estimated from the fractal dimension *D*, describing the surface complexity of a structure (Reichenbach et al., 1992b). Protoplasmic processes have surface:volume ratios in the order of 12 to 18 μm^{-1}, whereas fibrous processes have much lower surface:volume ratios of less than 4 μm^{-1} (Rasmussen, 1975; Wolff, 1976; Grab et al., 1983; Reichenbach et al., 1988, unpublished results). Velate processes seem to reach the highest surface:volume levels of 20 to 25 μm^{-1} (Rasmussen, 1975; Reichenbach et al., 1988, 1992b). Interestingly, there exists an indirect proportionality between the surface complexity (i.e., surface:volume ratio) and the length of glial cell processes (Siegel et al., 1991; Reichenbach et al., 1992b). This relationship may indicate that the absolute membrane surface area a glial cell can produce is limited (perhaps, by metabolic constraints).

A similar relationship exists concerning the diameter of radial glial cell processes; in a given species (and tissue) and at a given developmental stage, there is an inverse correlation between process length and diameter (Ugrumov et al., 1979; Bruni et al., 1983; Reichenbach and Wohlrab, 1986; Hanke and Reichenbach, 1987; Reichenbach et al., 1987) (Figure 4-14); the absolute diameter ranges between several microns and (in embryonic radial glia) less than 0.4 μm. When rat radial glial cells of very different lengths are compared at the same embryonic stage, their absolute volumes are found to be almost the same (Reichenbach et al., 1987). It seems as though ependymoglia require a certain period to produce a given cytoplasmic volume. Since they are attached to both surfaces of the neuroepithelium, they may be stretched differently by local tissue growth rates, but such elongation occurs at the expense of process diameter until new material can be synthesized. Such "passive" growth mechanisms have been studied by Jaeger (1988), and might be mediated by stretch-activated ion channels that have been demonstrated to occur in ependymoglial cells (Christensen, 1987). Generally, older cells have larger volumes than young cells (Reichenbach and Reichelt, 1986; Hanke and Reichenbach, 1987).

Rapid, reversible, protein synthesis-independent changes in cytoskeleton and cell shape are induced

by cyclic adenosine monophosphate (Ciesielski-Treska et al., 1984). They may be involved in some of the dynamic interactions with neuronal cells that are discussed in the next section.

Dynamic Properties: Functional Interactions with Neurons

Ultrastructural and even structural features of ependymoglial cells are by no means constant. Physiological stimulation of sensory epithelia and brain compartments evokes specific reactions among ependymoglia. In the olfactory epithelium for example, odorant stimulation evokes secretory activity by the sustentacular cells (Getchell et al., 1984). Stimulation by hormones or by exsiccation of neurons in the tuberohypophyseal system leads to changes in the covering of blood vessels by tanycytic endfeet (Wittkowski, 1973; Lichtensteiger et al., 1978), the ultrastructure of which changes with the physiological state (Rodríguez et al., 1979). In rhesus monkeys, the structure of hypothalamic tanycytes differs between male and female animals; moreover, the apical protrusions of tanycytes in females change their size and number depending on the estrous cycle (Knowles and Anand-Kumar, 1969). Disc shedding of photoreceptor outer segments occurs with a circadian rhythm, and is reflected by cyclic changes in phagocytotic metabolism of retinal pigment epithelial cells (cf. Steinberg, 1985). Adaptation to bright light causes retraction of pigment granules toward the basal pole in retinal pigment epithelium cells as well as in supporting cells of the reptilian parietal eye (Ali, 1971).

Furthermore, many lesions of neuronal tissue precipitate structural changes among the ependymoglia. In retinal detachment for example, the nuclei of Müller cells migrate out of the inner nuclear layer, and cells dramatically change their shape (Anderson et al., 1983). Examples of such structural adaptations of ependymoglial cell processes to changed microenvironments are also mentioned in the sections *Type I* . . . and *Type II* . . . above. In summarizing these few examples it may be stated that ependymoglial cells are continuously adaptating to the changing needs of the neuronal tissue.

DEVELOPMENTAL AFFINITIES OF EPENDYMOGLIA

In all vertebrates, the central nervous system arises from an ectodermal neural plate, and the peripheral sensory epithelia arise from ectodermal (or endodermal) placodes. By the time that the first neurons in the central nervous system become postmitotic, the tissue consists of a ventricular (or matrix) zone formed by cell nuclei undergoing interkinetic migration, and a marginal zone (*Randschleier*) formed by the neurites of first postmitotic neurons. The latter (Cajal-Retzius cells of neocortex, motoneurons of spinal cord, ganglion cells of retina, etc.) are found between both zones (in the "mantle zone"); some cerebrospinal fluid-contact neurons also seem to be generated early (retinal cone photoreceptors: Robinson, 1991; spinal cord sensory cells: Roberts and Clarke, 1982; Dale et al., 1987). At this stage, two types of radially oriented cells span the width of the epithelium: neuroepithelial stem cells and young radial glial cells (Levitt and Rakic, 1980; Eckenhoff and Rakic, 1984). Thus, the structure of the early vertebrate neuroepithelium closely resembles that of the primitive deuterostomian nervous system (see Figures 4-1 and 4-2).

Initially (Figure 4-15A) embryonic radial glial cells are not readily discriminable from neuroepithelial stem cells. Radial glial cells begin their morphological differentiation by sprouting lateral protrusions from their trunks (Figure 4-15B, C). Somewhat later, some of the cells begin to lose their contact to the ventricular system (Figure 4-15C) and, thus, are no longer members of the ependymoglial family (cf. Chapter 49).

It is difficult to determine when radial glial cells become committed from their neuroepithelial precursors, since their differentiation is rather conservative. Initially, there are no obvious morphological changes, and the cells continue to express a series of marker proteins that are already expressed by primitive neuroepithelial cells such as vimentin (Lemmon, 1986), nestin (Hockfield and McKay, 1985), antigen for RC1 antibody (Misson et al., 1988), intermediate filament-associated protein a-400 (Chabot and Vincent, 1990), and carbonic anhydrase (Linser and Moscona, 1982).

Mature ependymoglia (or ependymoglia-like cells) have sometimes been reported to act as precursor cells for neurons. This has been observed during regeneration of spinal cord (Egar and Singer, 1972), for neuromast sensory cells (Balak et al., 1990), cochlear hair cells (Corwin and Cotanche, 1988; Raphael, 1992), and also taste buds (Delay et al., 1986). Furthermore, neurogenesis from presumed ependymoglia has been reported in the brains of adult amphibians, reptilians (Polenov et al., 1972; Lopez-Garcia et al., 1988), and even birds (Alvarez-Buylla et al., 1990; cf. Alvarez-Buylla, 1990). These observations suggest that there is only a small (and often reversible) transition between neuroepithelial stem cells and radial glia.

In any case, radial glial cells characteristically are involved in the radial organization of young neurons within the nervous tissue. One such case is the olfac-

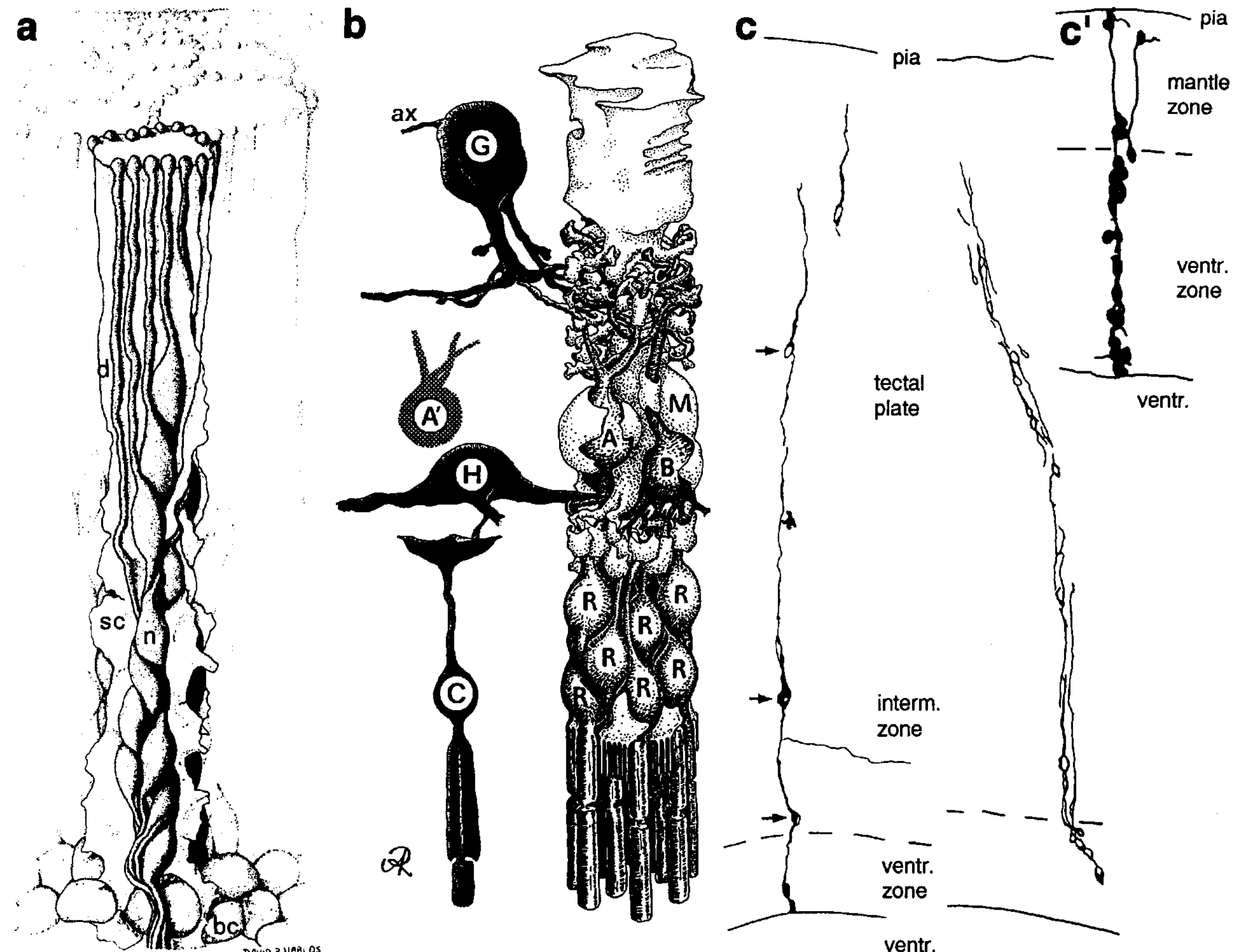


FIG. 4-16. The role of radial glial cells in organization of columnar units. (A) Adult rodent olfactory epithelium; shown is the columnar arrangement of the mature neurons (*n*) and their dendrites (*d*) around the supporting cells (*sc*); *bc*, basal cells that are the stem cells of the epithelium. (B) Adult rabbit retina; a columnar unit of neurons (*R*, rod photoreceptors; *B*, bipolar cells; *A*, amacrine cells) is ensheathed by cytoplasmic extensions from a Müller cell (*M*); these cells are probably the progeny of common precursor cell. Other types of neurons (*G*, ganglion cells; *H*, horizontal cells; *C*, cone photoreceptors, and some amacrine cells *A'*) are born earlier in ontogenesis, by another type of precursor cell. (C), (C')Camera-lucida drawings of cell clones in embryonic chicken optic tectum; each clone consists of one radial glial cell and up to 15 neuronal (or nonradial glial) cells that climb along the radial glial cell process. Retroviral vectors were introduced at *E4* (Figure C) or *E3* (Figure C') and clones demonstrated at *E10* (Figure C), or *E5* (Figure C'). [Figure A: from Graziadei and Monti Graziadei (1979), with permission; Figure B: from Reichenbach et al. (1993), with permission; Figures C and C': from Gray and Sanes (1992 and 1991), respectively, with permission.]

tory epithelium where receptor neurons are renewed from basal cells throughout life and form columnar units together with a supporting cell (Graziadei and Monti Graziadei, 1979) (Figure 4-16a).

In the mammalian retina, an early proliferative phase produces several types of "primary" neurons (black in Figure 4-16B). In a second proliferative phase, columnar clones are generated each from a common progenitor (white in Figure 4-16B); such a clone consists of one Müller cell and a defined number of rod photoreceptors, bipolar cells, and amacrine cells (e.g., Turner and Cepko, 1987; cf. Reichenbach et al., 1993). These neurons become ensheathed by lateral cytoplasmic excrescences of the Müller cell, and seem to form a metabolic and functional unit together with this cell (for detailed arguments, see Reichenbach et al., 1993). A similar phenomenon has been observed in the avian optic tectum (Gray and Sanes, 1991, 1992); progenitor cells resembling radial glial cells produce cell clones that consist of one radial glial cell and up to 15 neuronal (and nonradial glial) cells. The latter migrate along the radial glial process to reach the tectal plate (Figure 4-16C, C'). Obviously, this is a general mechanism in the mammalian central nervous system; see also Chapter 49.

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5 Microglial cells

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Microglial cell numbers are often said to range from 5 to 20% of the entire central nervous system glial cell population (e.g., Kreutzberg, 1987). As a conservative estimate, assuming that microglia represent 10% of the total glial cell pool and knowing that there are at least 10 times as many glial cells as neurons in the central nervous system, it is apparent that there are at least as many microglia as there are neurons. This simple calculation is astonishing only insofar as the very existence of microglial cells was questioned by some scientists until recently.

Research on microglia has grown significantly during the past decade. This positive trend can be attributed largely to the development of reliable histological methods for identifying the cells in tissue sections using light microscopy. Most of these methods, which are described in this chapter, can also be carried to the electron microscopic level, allowing verification of microglial identity through direct comparison of the presence of a specific marker with ultrastructural morphology. Before the advent of microglia-specific markers, electron microscopy was almost always needed for positive identification, using solely morphological criteria. An excellent description of the ultrastructural morphology of microglial cells can be found in Peters et al. (1991). An assortment of light microscopical techniques are now available that permit easy identification of microglial cells by neuroscientists from all backgrounds.

Regarding the terminology used in this chapter, I should point out that microglial cells in the adult central nervous system can assume at least three clearly identifiable states: (1) resting or ramified microglia, as are present in the normal, nonpathological central nervous system; (2) activated or reactive microglia that are found in pathological states but are nonphagocytic; (3) phagocytic microglia that represent full-fledged brain macrophages. Table 5-1 provides a summary of known microglial characteristics during these states as determined in rat brain. For more detailed descriptions the reader is referred elsewhere (Streit et al., 1988; Morioka et al., 1992a; Chapter 59, this volume).

HISTORICAL PERSPECTIVE

Following early descriptions of neuroglia by Virchow in the mid-nineteenth century, various other contemporary pathologists and psychiatrists, including such famous figures as Nissl and Alzheimer, commented on the possibility that the developing central nervous system was being invaded by cells of non-neuroectodermal origin. Speculation abounded as to the source of these invading cells, but with increasing consistency attention was being focused on the possibility that mesodermally derived cells were the invaders. Ultimately this led to the formulation by Ramón y Cajal (1913) of *el tercer elemento,* the third element of the central nervous system, referring to a group of cells that was morphologically distinct from both first and second elements (neurons and astrocytic neuroglia). Ramón y Cajal's third element, defined strictly in morphological terms, received further distinction into oligodendrocytes and microglia by Rio Hortega, the Spanish neuroanatomist who provided the first systematic investigation on microglial cells (Rio Hortega, 1932). Rio Hortega's detailed cytological observations, as well as his view of the origin of microglia, which gave rise to a controversy that has persisted until the present day, can be found in his original treatise and elsewhere (Theele and Streit, 1993; Chapter 10, this volume). The work of Rio Hortega remains relevant for most aspects of current microglial research.

METHODS FOR STAINING MICROGLIA

Silver Carbonate Method

The first selective stain for microglia was the weak silver carbonate method developed by Rio Hortega (1919). Despite its capriciousness, this method remained the only useful histochemical procedure for at least 50 years (Mori and Leblond, 1969). As with many other histochemical techniques involving metallic silver impregnations, Rio Hortega's weak silver carbonate method for microglia has very specific fixation requirements, which do not, however, guar-

antee reproducible results in every preparation. The results obtained are quite variable in terms of numbers of microglia stained, and vary also with the type of animal species used. For reasons unknown, the method seems to work reliably only in rabbit brain (Penfield and Cone, 1961). While it can be carried to the electron microscopic level (Mori and Leblond, 1969), its usefulness is limited by poor structural preservation and the deposition of metallic precipitates, which obscure much of the cellular detail.

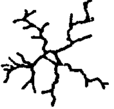
Enzyme Histochemical Methods

Thiamine pyrophosphatase (TPPase) and *nucleoside diphosphatase (NDPase)* are the most reliable and specific enzyme histochemical methods for staining resting microglial cells in a variety of species. These methods, which were developed by Novikoff and Goldfischer (1961), have been used successfully to localize microglia at both light and electron microscopic levels (Murabe and Sano, 1981; Schnitzer, 1989). Thiamine pyrophosphatase activity, originally localized to the Golgi apparatus in a variety of cell types, including neurons (Novikoff and Goldfischer, 1961), was later found to bè associated specifically with the plasma membrane of microglial cells and with blood vessels in the central nervous system (Murabe and Sano, 1981).

Nonspecific esterase is an enzyme commonly used to identify microglia in mixed brain cultures (e.g., Sawada et al., 1990). Nonspecific esterase staining is, however, of little use for detecting resting microglial cells in tissue sections. Actively proliferating, reactive microglial cells in the hypoglossal nucleus after peripheral nerve transection also do not show any staining for this enzyme (Schelper and Adrian, 1980). In contrast, nonspecific esterase can be found in microglia-derived brain macrophages that are prevalent in stab wounds. This supports the view that microglia isolated *in vitro,* which are nonspecific esterase-positive, are in fact microglia that have transformed into brain macrophages as a consequence of having been placed into tissue culture.

Activated and/or phagocytic microglia, *in vitro* as well as *in vivo,* show increased activities for a number of various other enzymes that are absent from resting microglial cells. These include acid phosphatase, 5'-nucleotidase, and oxidoreductase (reviewed by Oehmichen, 1980). More recent studies have also shown presence of nitric oxide synthase, lysosomal proteinases, plasminogen activator, lysozyme, purine nucleoside phosphorylase, and elastase (Castellano et al., 1990; Nakajima et al., 1992; Banati et al., 1993). It is worth noting that many of these enzymes are also found in other glial cells types and are there-

TABLE 5-1. *The Three States of Microglial Biology and Associated Characteristics*



	Resting	Activated	Phagocytic
Proliferation	–	+	+
Migration	–	+	+
Vimentin	–	+	+
Glial fibrillary acidic protein (GFAP)	–	–	–
Griffonia simplicifolia B_4-isolectin	+	++	++
Macrophage markers (ED1, ED2, OX-41)	–	–+	–+
CR3 complement receptor (OX-42)	+	++	++
MHC class I antigen (OX-18)	–	+	+
MHC class II antigen (OX-6)	–/+	+	+
CD4 antigen (W3/25)	–/+	+	+
Leukocyte common antigen (OX-1)	–	+	+

From Streit and Kreutzberg (1987), with permission.

fore not necessarily useful as selective histochemical markers for microglial cells.

Immunohistochemical Detection of Microglia

The microglial plasma membrane is complex, containing a large number of receptor and adhesion molecules, as well as enzymatic activities. This large repertoire of potentially immunogenic surface antigens has facilitated the generation of numerous antibodies that can be used in microglial staining procedures. Interestingly, many of the monoclonal antibodies that are presently in use were not produced with the intention of labeling microglia, but were generated originally through immunization against differentiation antigens found on cells of the immune system, such as macrophages, thymocytes, and lymphocytes. Following initial failures of demonstrating presence of some monocytic and lymphoid antigens on human microglia (Oehmichen et al., 1979), it was found later that a mouse macrophage-specific antigen could be localized on resting microglia with a monoclonal antibody designated F4/80 (Hume et al., 1983; Perry et al., 1985). These investigators also succeeded in showing the presence of Fc and complement receptors on resting mouse microglia using antibodies 2.4G2 and Mac-1, respectively. Similarly, ramified microglia in rat brain can be demonstrated reliably using the OX-42 antibody against the CR3 complement receptor (Graeber et al., 1988a). It is important to note that these receptors are also found on macrophages in nonneural tissues, which underscores the phagocytic potential of microglia, as well as their close relationship to the myelomonocytic cell lineage. Cross-reactivity of the antibodies with microglia and blood monocytes has been interpreted to indicate that microglia are derived from monocytes. However, this interpretation should be considered cautiously, since both microglia and monocytes are fully differentiated cell types, and there is no unequivocal evidence to indicate a direct lineage relationship between the two cell types (see Chapter 10).

Microglia in human brain can be visualized using analogous antibodies against typical macrophage surface receptors (Akiyama and McGeer, 1990). In addition to the expression of Fc and complement receptors, other cell adhesion molecules are expressed constitutively on resting microglia in normal brain. Belonging to the integrin superfamily of adhesion molecules, these include typical lymphocytic antigens, such as lymphocyte function antigen, CD4 antigen, as well as leukocyte common antigen (Perry and Gordon, 1987; Akiyama and McGeer, 1990). Species differences between mouse, rat, and human in the constitutive expression of these molecules on resting microglia have been described, and these are likely due to both antibody specificities, as well as variations in tissue processing techniques. B-lymphocyte antigens are also detectable on human microglial cells using monoclonal antibodies of the LN series, in particular LN-1 and LN-3 (Miles and Chou, 1988; Dickson and Mattiace, 1989; Sasaki et al., 1991). While the LN-1 antibody may label both astrocytes and microglia depending on fixation and tissue processing techniques, antibody LN-3 appears to have an exclusive specificity for microglia in both the normal and pathological human brain (Figures 5-1 to 5-3). Thus, the microglial surface membrane bears molecules that are usually associated with white blood cells. Consistent with this, is also the localization of antigens of the major histocompatibility complex (MHC) on microglia. Until a few years ago it was thought that MHC antigens were entirely absent from brain, thereby supporting the conception of the brain as an immunologically privileged organ. However, it is now well documented that the normal brain does indeed contain MHC antigens, and that the principal cell type expressing MHC antigens is the microglial cell (Craggs and Webster, 1985; Hayes et al., 1987; Streit et al., 1989). The constitutive expression of MHC antigens in normal brain is, however, not limited to microglia, but includes endothelial cells, as well as certain cell types located in the wall of cerebral blood vessels, and there are considerable species differences in the levels of constitutive MHC antigen expression on these various cell types. MHC antigen expression is much increased in the presence of pathology and after systemic administration of gamma interferon (McGeer et al., 1988; Steiniger and van der Meide, 1988; Streit et al., 1988; Chapter 59, this volume).

In addition to known monocytic and lymphoid differentiation antigens, various other and yet to be identified antigens have been detected on the microglial membrane through recently developed monoclonal antibodies (Imamura et al., 1990; Gehrmann and Kreutzberg, 1991). Using Western blot analyses, these studies have demonstrated antigens with molecular weights of 46, 62, 70, 78, 95, and 116 kD, thus emphasizing the multiantigenicity of the microglial surface. Another example of this can be found in the recent study by Flaris et al. (1993), which describes the generation of a large panel of new antimicroglial antibodies. However, despite these efforts, a truly specific antibody for microglia, that is, one that does not cross-react with other macrophages in extracerebral tissues, has not been generated to this date. As a general rule, it can be said that any antibody that binds to ramified (resting) mi-

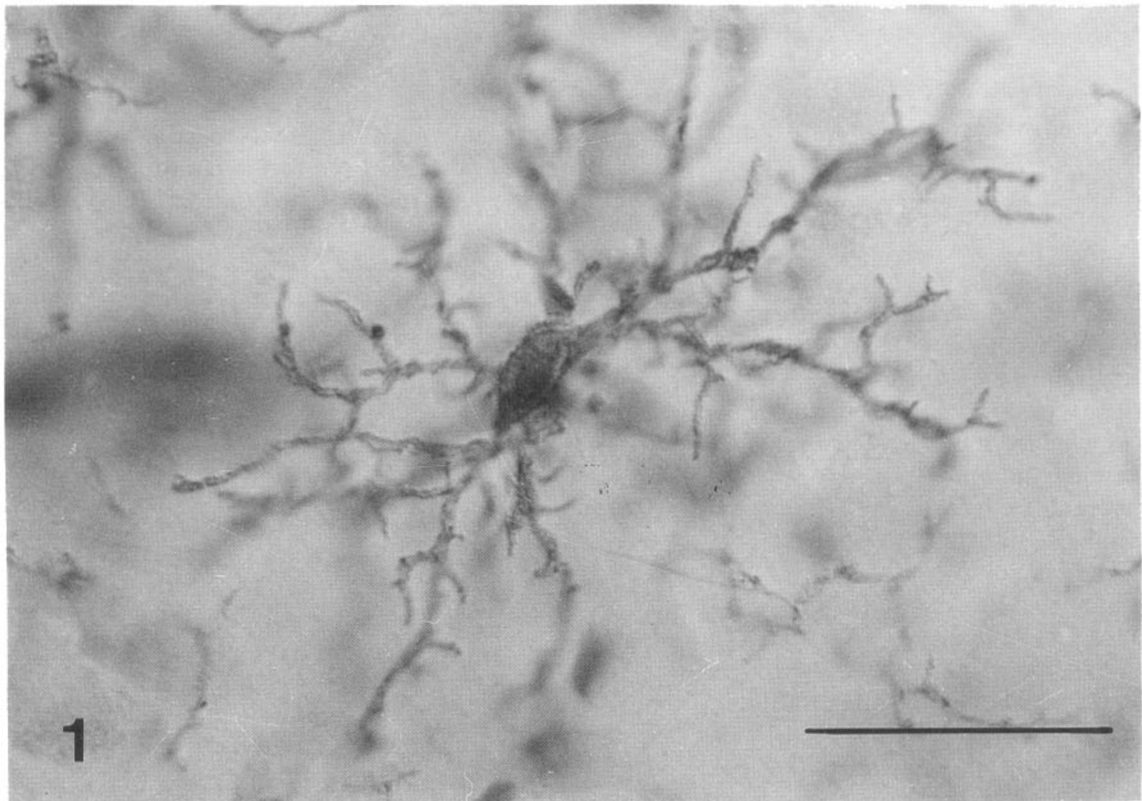


FIG. 5-1. Ramified resting microglial cell in human brain visualized with monoclonal antibody LN-3. Note extensive branching of cytoplasmic processes Bar = 30 μm.

croglial cells will also stain activated microglia and microglia-derived brain macrophages. Conversely, an antibody that stains brain macrophages does not necessarily stain resting microglia. This suggests that either the antigenic repertoire of resting microglia is smaller than that of brain macrophages, or that the level of expression of certain antigens in resting cells is below the detection limit of the immunohistochemical procedure. As an example of this, consider the expression of the intermediate filament protein vimentin. Resting microlia are vimentin-negative, but they quickly upregulate vimentin expression as part of their activation program in response to neuronal injury (Graeber et al., 1988b). Thus, vimentin expression is transient, being evident only in activated microglia and brain macrophages.

Immunohistochemical localization of ramified microglia has been achieved through the use of phosphotyrosine antibodies (Tillotson and Wood, 1989). This procedure detects the products of an enzymatic reaction carried out by tyrosine kinase. There are important functional implications for this observation, since it is known that tyrosine kinases are often associated with cell surface receptors, which are plentiful on the microglial membrane, as discussed above. Various other immunohistochemical methods designed to detect somewhat unconventional antigens, such as vaults and ferritin have also been described (Kaneko et al., 1989; Chugani et al., 1991). Vaults, which are multiarched ribonucleoprotein particles of unknown function, appear to be enriched in microglia during ontogeny, but largely disappear in the adult stage. Ferritin, on the other hand, is a well-known iron-storage protein, and its detection in microglia suggests their active participation in iron metabolism, as in the case of blood monocytes and other tissue macrophages.

In summary, tremendous progress has been made in the last few years with regard to developing immunohistochemical procedures for the detection and identification of microglia. These methods promise to be developed further in the years to come, and they will undoubtedly play a critical role for the continued study of microglial functions.

Lectin Histochemical Detection of Microglia

During the course of investigations examining the distribution of complex carbohydrates in nervous

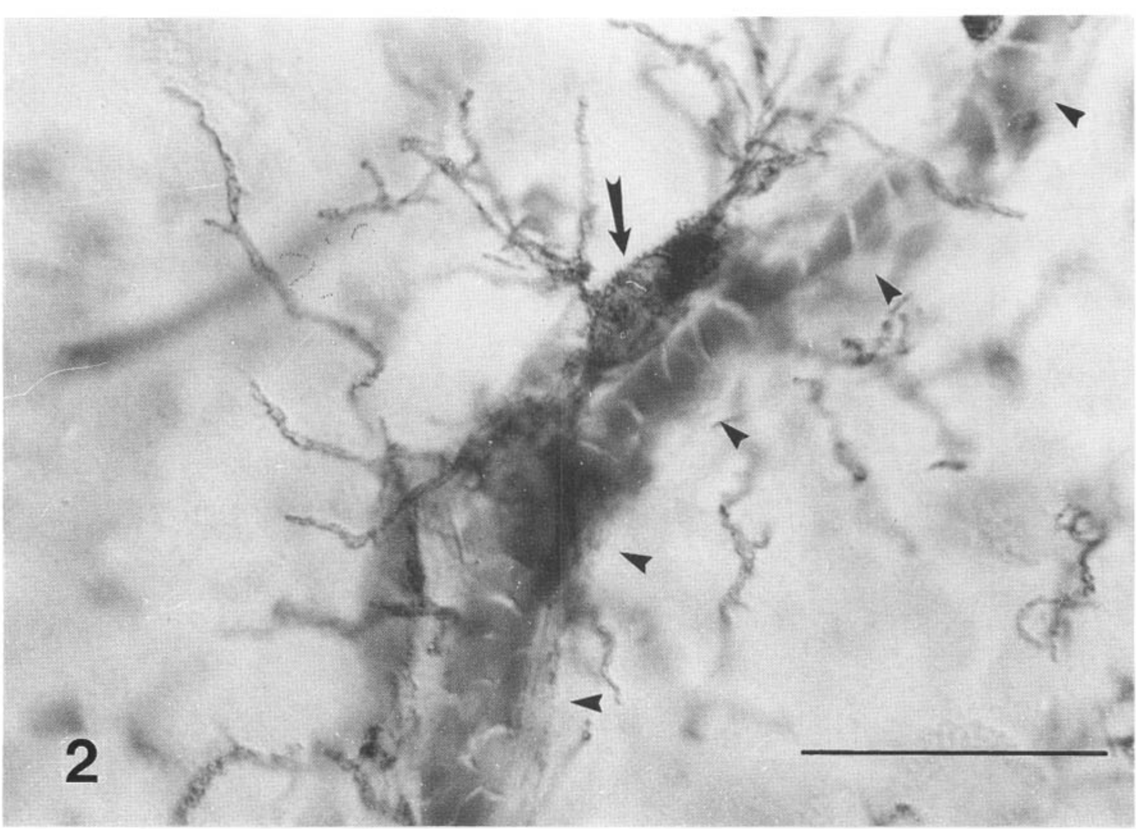


FIG. 5-2. Ramified resting microglial cell in perivascular position (*arrow* points at nucleus). The course of the blood vessel, which is filled with red blood cells, is indicated by the *arrowheads*. LN-3, human brain. Bar=30 μm.

tissue with lectin histochemistry, it was noted that the B_4-isolectin derived from *Griffonia simplicifolia* resulted in the selective visualization of a population of glial cells, which were tentatively identified as microglia (Streit et al., 1985) (see Figure 5-4). These observations were confirmed soon thereafter in human tissue where it was shown that the lectin from *Ricinus communis* could be used as a histochemical marker for microglia (Mannoji et al., 1986). Both lectins have similar sugar binding characteristics in recognizing anomeric forms of galactose: *Griffonia simplicifolia* binds to α-D-galactose and *Ricinus communis* recognizes β-D-galactose residues. Another β-D-galactose-binding lectin derived from mistletoe has been shown to preferentially stain human over rat microglia (Suzuki et al., 1988), stressing the high degree of specificity of lectin binding. Thus, there appears to be only a slight difference in glycocalyx composition between rodent and human microglial cells, being simply one of anomeric configuration. The galactose sugar residues occur as terminal sugars in the oligosaccharide side chains of nervous system glycoproteins, which are embedded in the microglial plasma membrane, as revealed by electron microscopy (Streit and Kreutzberg, 1987). (Figures 5-5 and 5-6). The nature and function of lectin-binding glycoproteins on microglial cells has not yet been resolved. However, it is possible that some of the surface antigens and receptors described in the preceding section might be responsible for lectin binding. In fact, most cell surface receptors show some degree of glycosylation. Lectin staining is perhaps the easiest and most reliable method for visualizing microglia in tissue sections, since the carbohydrate epitopes, unlike most proteins, are relatively resistant to chemical alterations by fixation and tissue processing techniques.

Other Methods for Labeling Microglia

Unlike most cells in the mature central nervous system, microglia are easily induced to proliferate which makes them susceptible to labeling with [^{3}H]thymidine, or any other marker of dividing cells, such as 5-bromo-2′-deoxyuridine. Microglial proliferation is usually triggered by some disturbance in central nervous system homeostasis, such as neuronal injury (Kreutzberg, 1966), but there is also evidence to suggest that microglial proliferation occurs

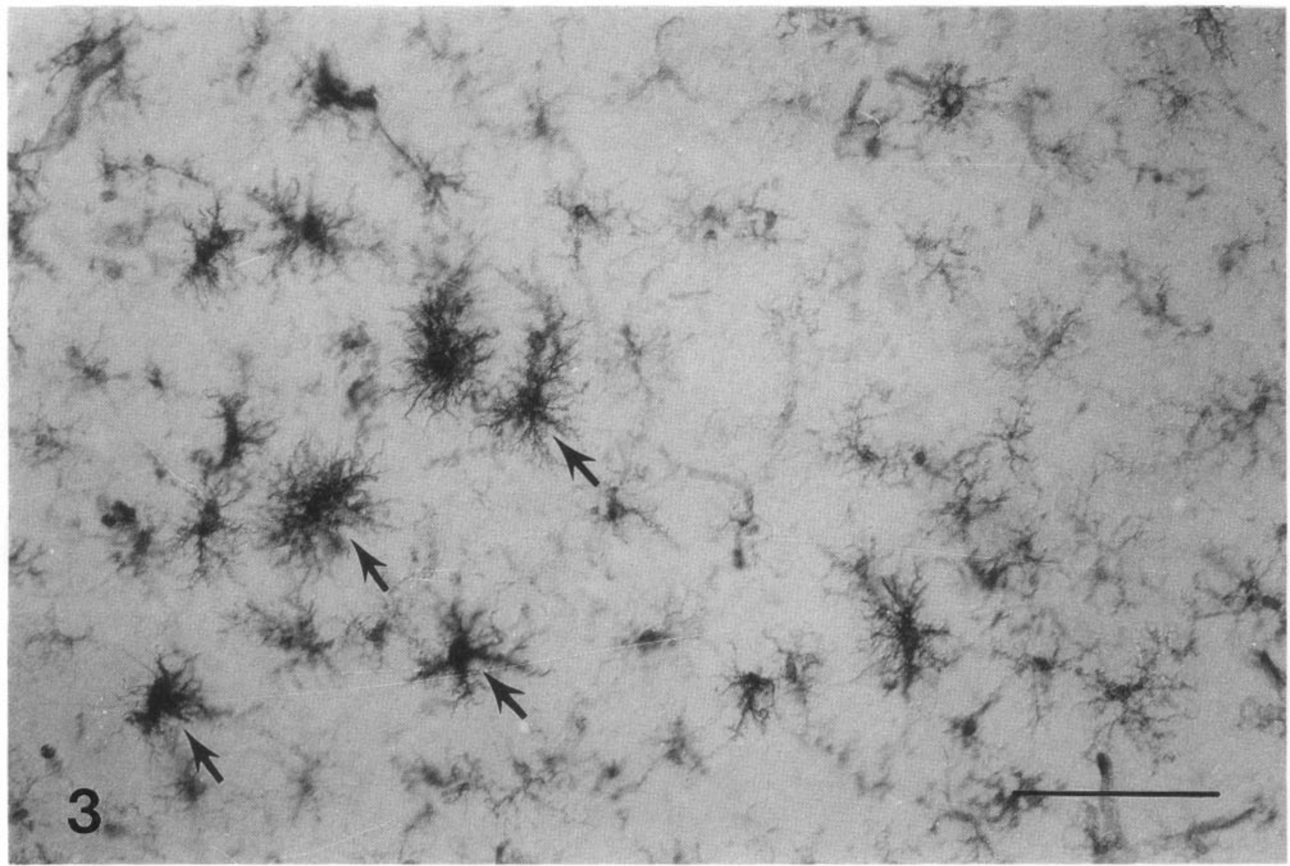


FIG. 5-3. Activated microglial cells in human brain stained with LN-3 (*arrows*). Compared to resting cells, activated microglia show enhanced staining and have a bushy appearance. Bar=100 μm.

normally in the rat brain, albeit at low levels (Korr et al., 1983). Microglia may also be labeled directly or indirectly using various dyes and tracer substances. An example of a direct approach is the intraperitoneal injection of the fluorescent dye, rhodamine isothiocyanate, which results in the labeling of ameboid microglia in the corpus callosum. The rhotamine isothiocyanate-labeled ameboid cells were observed subsequently to transform into rhodamine isothiocyanate-labeled ramified microglial cells (Leong and Ling, 1992), thus confirming earlier observations using colloidal carbon introduced in the form of India ink (Ling et al., 1980). An indirect method for labeling microglia makes use of their ability to phagocytose dead or dying neurons. Following injection of the appropriate tracer substance into nerve axons, the tracer is transported retrogradely toward the parent neuronal cell bodies. If the injected nerve is also axotomized, in many instances this will cause degeneration of the parent neurons, followed by removal of dead neurons through locally present microglial cells which, will phagocytose not only the neuronal debris but also the tracer substance and thus become labeled. Such experiments have been successfully carried out in various systems, including the visual system resulting in the labeling of retinal microglia with the carbocyanine dye DiI (Thanos et al., 1991), the dorsal motor nucleus of the vagus where the neural tracer Fluoro-Gold was used (Rinaman et al., 1991), and also in the rat facial nucleus where Fluoro-Gold was used in conjunction with toxic ricin to induce motor neuron degeneration (Streit and Graeber, 1993).

MICROGLIA AND RELATED CELL TYPES

Definitions

Microglial cells, because they can change their morphology and appearance in certain pathological and developmental states, have been given various descriptive names and attributes, such as *rod cells, gitter cells, globoid and ameboid cells,* to name a few. Even though these terms accurately reflect the cells' changed appearance, the constantly changing terminology has somewhat obscured the true identity of the cells associated with the term microglia. In the

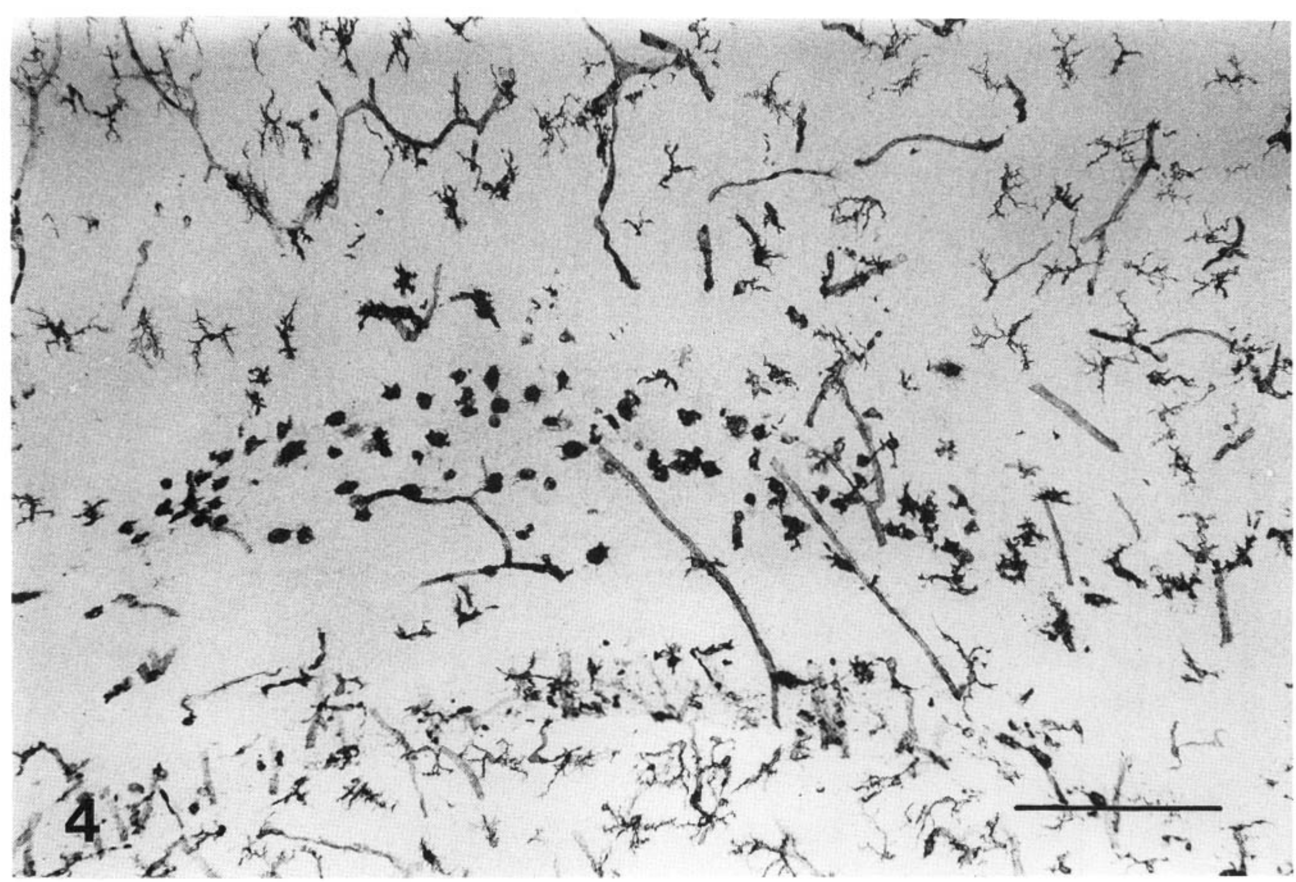


FIG. 5-4. A cluster of round ameboid microglial cells in the supraventricular corpus callosum of the developing rat brain is shown on postnatal day 7 (*center*). Note that increasingly differentiated (ramified) microglial cells surround the central cluster of ameboid cells. Microglia are visualized using the B_4-isolectin from *Griffonia simplicifolia*. Bar=200 μm.

normal and nonpathological brain, resting microglia can be defined both in terms of morphology and phenotype. They are highly branched (ramified) glial cells with a small amount of perinuclear cytoplasm and a small, dense, and heterochromatic nucleus (Figure 5-1). They can be distinguished easily from other glial cells by their surface immunophenotype, that is, they are the only glial cell type which constitutively expresses the CR3 complement receptor (recognized by monoclonal antibody OX-42 in the rat) and binds lectins with a specificity for galactose residues. Furthermore, at the ultrastructural level microglia are recognizable as true parenchymal constituents of the central nervous system, that is, they are located outside of the vascular basement membrane. At the same time, they may be considered part of the perivascular glia limitans, since microglial cytoplasmic processes are found intermingled with the layer of astrocytic foot processes (Lassmann et al., 1991). The observation that microglia are frequently found in the vicinity of blood vessels has resulted in the use of the term *perivascular microglia,* which is yet another descriptive term referring to parenchymal microglial cells, as defined above, which happen to be located near a cerebral blood vessel (Figure 5-2). "Perivascular" microglia are not to be confused with the so-called *perivascular cells,* which, unlike microglia, are *not* part of the central nervous system parenchyma, since they are enclosed by a perivascular basement membrane. Thus, perivascular cells are cellular components of the vascular wall which fit the morphological definition of pericytes (Peters et al., 1991; Mato et al., 1986; Graeber and Streit, 1990a). However, like microglia, perivascular cells are phagocytic and may express MHC antigens (Mato et al., 1986; Streit et al., 1989), which has made it difficult in certain pathological situations to distinguish between perivascular cells and "perivascular" microglia. In the normal brain these two cell types are readily distinguished by their morphology and surface immunophenotype. Perivascular cells are seen only in association with blood vessels, they are not ramified, but rather of an elongate shape, and they can

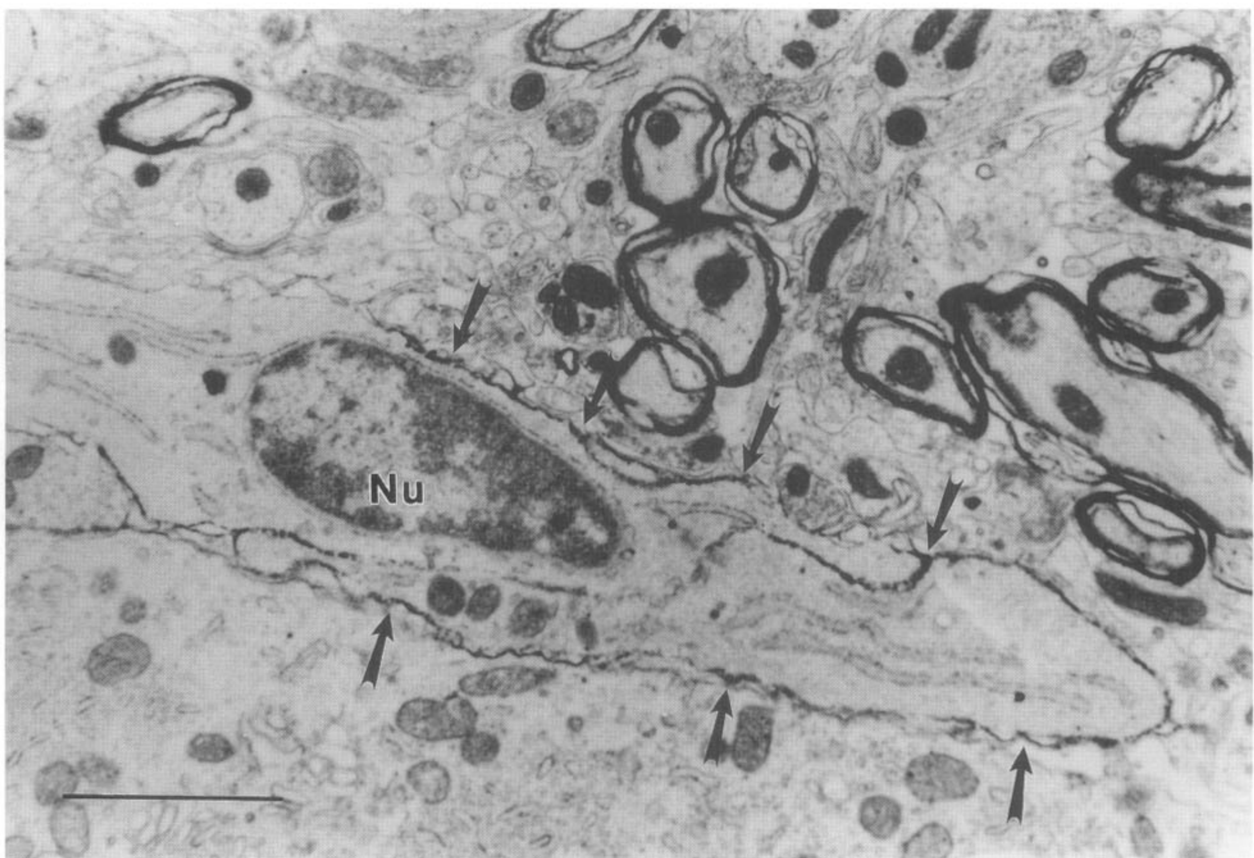


FIG. 5-5. Ultrastructural appearance of a microglial cell in rat brain. The cell has a small heterochromatic nucleus (*Nu*). Its plasma membrane (*arrows*) is accentuated by staining with the *Griffonia simplicifolia* B_4 isolectin, which binds to carbohydrate side chains of membrane glycoproteins. The cytoplasm contains prominent cisternae of rough endoplasmic reticulum. Bar=2 μm. From Streit and Kreutzberg (1987) with permission.

be specifically labeled in the rat with an antibody designated as ED2 (Graeber et al., 1989a). Thus, at least two clearly definable and indigenous sources of brain macrophages are present in normal brain: microglia and perivascular cells. *Brain macrophage* is a general term that encompasses all phagocytic cells, including blood-derived monocytes, which may enter the central nervous system following a lesion that causes a disruption of the blood-brain barrier.

Distribution, Morphology, and Phenotype

Microglia are distributed throughout the normal central nervous system with regional differences having been reported in mouse brain (Lawson et al., 1990). According to these authors, the highest microglial densities are encountered in the hippocampal formation, the olfactory telencephalon, portions of the basal ganglia, and the substantia nigra. A total number of 3.5×10^6 microglia is estimated to reside in the adult mouse brain (Lawson et al., 1990). Individual microglial cells typically occupy distinct territories, such that cytoplasmic processes of neighboring cells do not make contact with each other. The morphology and branching patterns of microglial cells show heterogeneity between different brain regions, and this is perhaps most remarkable when comparing cells in gray and white matter. Microglia in gray matter tend to be profusely ramified with processes extending into all directions, while cells in the white matter often align their cytoplasmic extensions in parallel, but also at right angles, to nerve fiber bundles. Thus, the shape of microglial cells adapts well to the architecture of the brain region they populate. Microglial phenotype, in contrast, appears to be influenced by the chemical composition of the microenvironment. For example, MHC class II-positive, as well as CD4-positive microglia are localized preferentially in white matter of normal brain (Hayes et al., 1987; Perry and Gordon, 1987; Streit et al., 1989; Mattiace et al., 1990). Brain regions lacking a blood-brain barrier, such as the cir-

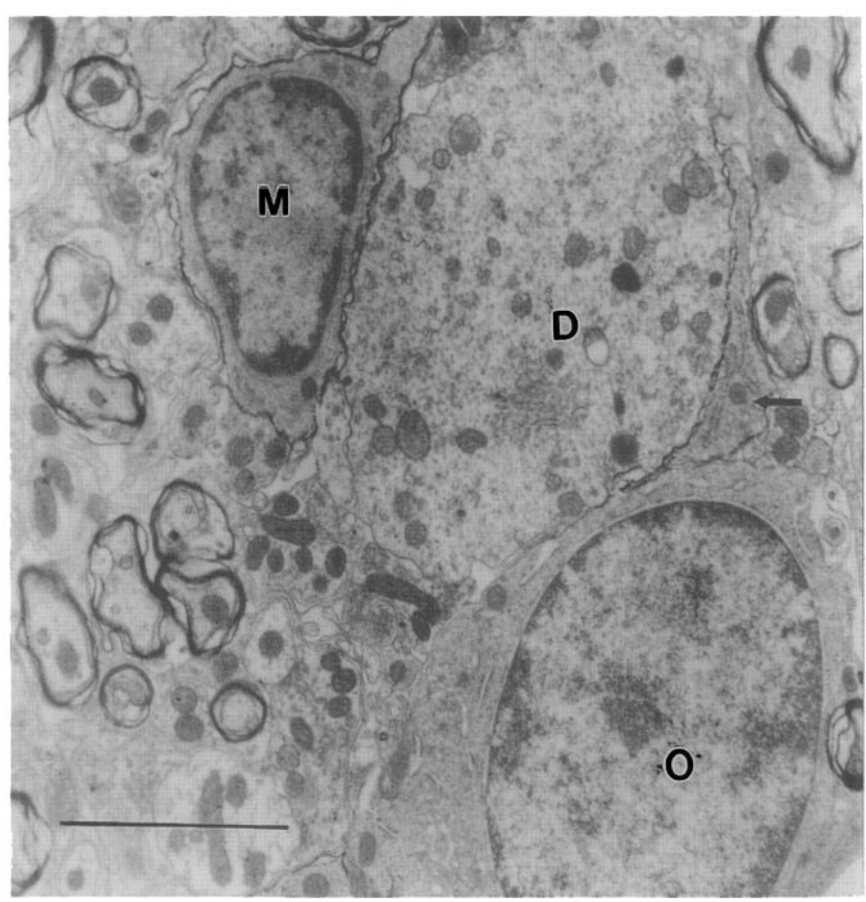


FIG. 5-6. The electron micrograph shows a microglial cell (*M*), an oligodendrocyte (O), and a large dendrite (*D*). Both cell types have a heterochromatic nucleus which is larger in the oligodendrocyte. Note lectin staining of the microglial plasma membrane (as in Figure 5-5). A cell process of the microglial cell is wrapped around the dendrite and appears on the other side (*arrow*). Bar=2 μm. From Streit and Kreutzberg (1987), with permission.

cumventricular organs, do show microglia and microglia-like cells, such as the Kolmer cells of the choroid plexus, with a different phenotype, suggesting that serum proteins influence microglial phenotype (Perry et al., 1992). This is supported further by *in vivo* studies showing profound changes in microglial phenotype after brain lesions that compromise the blood-brain barrier, such as forebrain ischemia and kainic acid injections (Morioka et al., 1992b; Finsen et al., 1993). Similar changes in microglial immunophenotype occur also when the cells are maintained *in vitro* using serum-containing culture medium (Graeber et al., 1989b).

Microglia in Tissue Culture

Although the maintenance of microglia-brain macrophages in tissue culture is a technique that was already in use during the early 1900s, and possibly even earlier, the *in vitro* approach has regained significant popularity during the 1980s. The technique described by Giulian and Baker (1986) is now widely used with numerous modifications. When culturing microglial cells, perhaps more so than with any other neural cell type, it becomes immediately apparent that microglia *in vitro* are very much different from microglia *in vivo,* and that they closely resemble cultured macrophages. The preparation of primary mixed brain cultures, from which microglia are isolated, causes the generation of huge amounts of tissue debris. This, together with a required high serum content of the growth media promotes more or less instantaneous transformation of microglial cells into microglia-derived brain macrophages. Although it has been reported that prolonged maintenance of isolated microglia in lipopolysaccharide-free media (Gebicke-Haerter et al., 1989) can result in the formation of branched cells *in vitro,* the extensive ramification of microglial processes as it is seen in tissue sections is rarely achieved in the culture dish.

However, and despite these difficulties, tissue culture studies of microglia during recent years have contributed substantially to our current understanding of microglial biology. The most significant contribution that has come from the *in vitro* experiments is the demonstration that microglia secrete a number of cytokines, such as interleukin 1 (Giulian et al., 1986), interleukin 6 (Frei et al., 1989), and tumor necrosis factor (Sawada et al., 1989). Tissue

culture studies have additionally revealed microglial responsivenes to growth factors, such as GM-CSF and CSF-1, which are potent inducers of microglial proliferation (Giulian and Ingeman, 1988; Suzumura et al., 1990). Microglial secretion of, and their responsiveness to, cytokines-growth factors is currently under intense investigation, and it can be anticipated that future studies will reveal additional cytokines-growth factors involved in mediating microglial functions. The demonstration that microglia are highly secretory cells has provided a conceptional complement to the *in vivo* studies that have focused on immunophenotypic plasticity of surface molecules. Thus, *in vitro* and *in vivo* approaches have uncovered secretory and surface-bound molecules, respectively, both of which are necessary to facilitate microglial functions. The two experimental approaches have converged into a concept that now views microglia as the local immune system of the brain (Graeber and Streit, 1990b).

Microglia in Different Species

While microglia are best studied in rodents and human, primates and most other mammalian species are known to have microglia. Since species-specific antibodies for localizing microglia in cat, dogs, and primates are not yet available, the aforementioned enzyme and lectin histochemical methods are likely to be feasible alternatives. Recently, we have been able to stain microglial cells in the brain of the Florida manatee using the B_4-isolectin from *Griffonia simplicifolia*. The fact that the same lectin can also be used for staining microglial cells in an invertebrate species, namely, the leech (McGlade-McCulloh et al., 1989), strongly suggests that the glycoconjugates recognized by the lectin represent evolutionary conserved molecules. Similarly, NDPase histochemistry has been used for visualizing microglia in nonmammalian species, such as chicken and lizard (Fujimoto et al., 1987; Castellano et al., 1991). A recent report describes immunohistochemical staining of microglia in *Oreochromine* fish, using a new monoclonal antibody directed against fish macrophages (Dowding et al., 1991). The study also suggests that fish microglia, just like their mammalian counterparts, are capable of proliferating after nerve injury.

MICROGLIAL FUNCTIONS AND FUNCTIONAL PLASTICITY

The term functional plasticity was introduced to emphasize the enormous malleability of microglial cell shape and immunophenotype seen under pathological conditions (Streit et al., 1988). While specific aspects of microglial activation in pathological circumstances are discussed in Chapter 59 of this book, I should like to note, in a general sense, that following a pathological insult microglial cells tend to become hypertrophied with stout processes and may take on a bushy appearance (Figure 5-3). If neuronal degeneration is present, they may continue their metamorphosis and subsequently transform into round brain macrophages.

In the normal brain, such microglial plasticity is encountered during development. Although the origin of ameboid microglia is still a matter of dispute, there is little doubt that, during development, round ameboid cells differentiate to become the highly ramified resting cells seen in the adult. In support of this process is the presence of increasingly ramified microglia in the vicinity of ameboid microglial cell clusters that are prevalent in various white matter regions of the young postnatal rodent brain (Figure 5-4). Thus, structural plasticity of microglia during development and during pathology proceed in opposing directions with cell morphology becoming more ramified during ontogeny, and less ramified during pathology. While this may seem trivial, it nevertheless serves to underscore the direct relationship of cell shape to function, hence the term functional plasticity. We already know that these morphological transformations are accompanied by substantial changes in cell phenotype, and it is virtually certain that they are also accompanied by changes in cytokine production and secretion. However, the latter are still subject to ongoing investigations. Thus, we hope to soon be able to define functional plasticity in terms of both membrane-associated, as well as secretory activity. As to well-defined microglial functions in the adult, these are for the most part related to microglial activity in the diseased brain and include antigen presentation, cytotoxicity, neovascularization, and phagocytosis. Some of these functions are also important for the developing brain, in particular, phagocytosis of dying neurons. For example, naturally occurring cell death is prominent in the developing retina, and dying retinal ganglion neurons are removed by microglial cells (Hume et al., 1983). Although it has been suggested that retinal ganglion cell death serves to attract bloodborne macrophages to populate the retina and differentiate into microglia, this hypothesis seems incompatible with the fact that microglia are present in the rodent retina several days before the onset of ganglion cell death (Ashwell et al., 1989).

In the normal adult brain, microglial functions are still ill-defined; however, it would be safe at this time to propose that microglia serve a major role in the

immune surveillance of the central nervous system. Their ubiquity and regular spacing throughout the brain and spinal cord, as well as their morphology and phenotype, strongly indicate their function to be one of specialized immunocompetent cells guarding one of our most vital and vulnerable organs.

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6 Morphology of nonmammalian glial cells: functional implications

N. JOAN ABBOTT

HISTORICAL PERSPECTIVE

Insights from Studies of Nonmammalian Glial Cells

This volume considers the neuroglial cells of many animal groups. The desire to understand the functions of the human brain and its pathologies has meant that much work has been done on mammalian preparations. However, studies on nonmammalian glial cells can give information not available from the study of mammalian glia alone. This chapter summarizes the morphology of nonmammalian glial cells, concentrating on examples where the structural information contributes to understanding of function. Certain animal groups, such as insects, fish, amphibia, and birds, are particularly convenient for the study of glia during development because of the transparency or accessibility of the embryo. The large glial cells of certain invertebrates and lower vertebrates have proved particularly convenient for biochemical and electrophysiological study. By surveying glial morphology and function in a range of animal groups it is possible to present a picture of the evolutionary pressures that have shaped the arrangements seen in higher forms. Studies of the morphology of neuroglia thus give insights into the embryology, physiology, and evolution of neuroglial cells.

Definition and Classification of Nonmammalian Glia

Although the first anatomical studies of glia by Virchow, Golgi, and Ramón y Cajal between 1856 and 1920 used mammalian material, a comparative approach was being applied even during this period. The first tentative identifications were based on histological appearance and resemblance to mammalian glia. Thus in the cephalopod optic lobe, Lenhossèk (1896) described "bushy glial cells," and Ramón y Cajal (1917) described "epithelial cells" around neurons and "protoplasmic astrocytes" throughout the lobe. The leech (annelid) was of major medicinal interest, and its glia were described by several eminent histologists, including Rio Hortega and Retzius (see Ito, 1936).

There are two chief problems in describing nonmammalian glia: first, the definition of what constitutes a "glial cell", and second, the classification of glial subtypes. Some authors used the term "glia" for all cells within the boundary of the nervous system that were not clearly neuronal (Clayton, 1932; Hess, 1958). However, this classification frequently included cells derived from blood vessels, connective tissue, and mesenchymal elements. Radojcic and Pentreath (1979) proposed that the term "glia" should be restricted to nonneuronal cells (1) originating from embryonic ectoderm, and (2) having an intimate morphological relationship with neurons (sheathing, investing), or separating the neuronal compartment from mesodermal elements (e.g., at the vascular interface). It is not always possible to apply these criteria rigorously, since it can be difficult to distinguish glia from neurons, and embryonic information is often lacking, but they will form a working basis for the present account.

Invertebrate glia show a variety of morphologies depending on species and location. Glial cells appear to be absent in the lowest groups, the Mesozoa, Porifera, Coelenterata, and Echinodermata, but present in Platyhelmintha, Aschelmintha, Annelida, Arthropoda, and Mollusca (reviewed by Radojcic and Pentreath, 1979) (Figure 6-1). As invertebrates do not have true compact myelin, although some show periaxonal glial wrappings (Kirschner and Blaurock, 1992), no class of cell equivalent to oligodendrocytes can be identified. Moreover, immunocytochemical markers for astrocytes fail to cross-react with invertebrates in most cases, so it has not been possible to define a class of invertebrate glial cell equivalent to vertebrate astrocytes. Instead, invertebrate glia have been described according to their position (e.g., *sheathing, perineuronal, neuropilar, gliovascular*) or morphology (*plasmatic, protoplasmic, fibrous, Schwann-like*) (Radojcic and Pentreath, 1979). Of

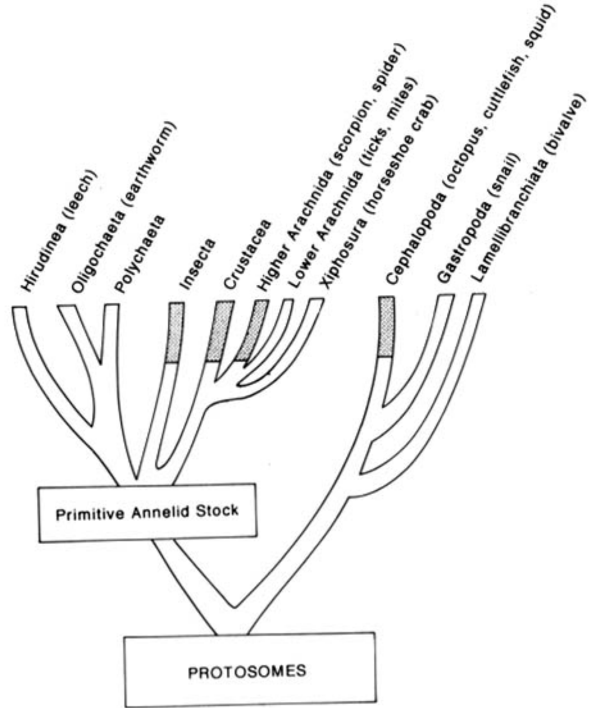


FIG. 6-1. Evolutionary tree of higher invertebrates, with modern groups at the top. Groups with a glial blood-brain barrier are shown (*shaded*). [Basic tree after Walker and James (1980).]

the commonest forms, *protoplasmic glia* have short thick processes, while *fibrous glia* have long thin ones; both may contain intermediate filaments.

Advantages of Nonmammalian Glia as Experimental Preparations

The modern interest in glial cell physiology began in the early 1960s (reviewed in Nicholls et al., 1992). The glial cells of invertebrates and lower vertebrates have some advantages for physiological study, chiefly their size and accessibility. Examples are the giant glia of the leech abdominal ganglia suitable for studies of electrophysiology and metabolism, the glial *perineurium* forming the insect blood-brain barrier, and the Schwann cells of the cephalopod peripheral nerve. Where comparison between invertebrate and vertebrate glia has been possible, several common properties have emerged, showing that studies in a range of animal groups can add to our understanding of glial architecture and function.

Structure and Classification of Glial Cells in Relation to Function

The earliest histologists were content to describe the position of glial cells and to categorize them on morphological criteria. As physiological and pharmacological studies progressed, it became obvious that the function of glial cells was closely related to their morphology. Thus glia associated with vertebrate neurons develop specific associations with axons (oligodendrocytes and Schwann cells forming internodal membrane wrappings, astrocytes sending processes to the nodal regions) and dendrites (enclosing groups of synapses in "glomeruli"), and glial cells at the blood-brain interface either form a sheath of endfeet on the vessel wall (most higher vertebrates) or themselves form an epithelium-like barrier (elasmobranchs and higher invertebrates). The elongated processes of glial cells in the retina and the syncytial nature of the glial network in vertebrate brain may play important roles in spatial redistribution of potassium (see Chapter 37). Any classification that does not consider function is likely to be unsatisfactory. This chapter reviews the morphology of nonmammalian glial cells, highlighting features that contribute to glial physiology and, where possible, giving a classification that incorporates functional information.

INVERTEBRATE GLIAL CELLS

Organization of Invertebrate Nervous Systems

Metameric invertebrates (including annelids, insects, and Crustacea) have a segmental body plan, reflected in the organization of the central nervous system as a series of segmental neural ganglia, linked by axons running in "connectives," with some fusion of ganglia in regions such as the head and thorax (Bullock and Horridge, 1965). In mollusks, most ganglia are in the region of the head, with a few outliers in the tissues. Most invertebrate ganglia do not have an internal vasculature, although intraganglionic vessels are found in higher annelids, Crustacea and cephalopod mollusks. Invertebrate glial morphologies reflect this diversity of organizational pattern.

Annelids

The Large Glia of the Leech Central Nervous System Surround Groups of Neurons. The importance of the leech in medicine made it an obvious choice for studies on the anatomy and embryology of the nervous system, and the leech segmental ganglia were described in detail by histologists Ramón y Cajal, Rio Hortega, and Retzius (reviewed in Muller et al., 1981), and more recently with the electron microscope (E. Gray and Guillery, 1963; Coggeshall and Fawcett,

1964). Each segmental ganglion is less than 1 mm in diameter, contains around 400 neurons, and is quite transparent. The neurons are organized in a stereotyped "map" with distinctive positions, shape, branching patterns, and physiology. A small number of large glial cells (*packet glia, neuropil glia, connective glia*) fill the spaces between the neurons and axons. Groups of neuron cell bodies and their initial axonal processes are embedded within a single large (packet) glial cell (Figure 6-2), while in the connectives many hundreds of axons are surrounded by another large glial cell several millimeters long. Four large ectodermal precursor cells (N, O, P, and Q teleoblasts) are responsible for the production of all the cells of the adult ganglia. As also recently established for vertebrate neural development, the earliest precursors give rise to both neurons and glial cells. Thus the N teleoblast generates the neuropil glial cell as well as the Retzius neuron, a sensory neuron specific for touch and nociception (Kramer and Weisblat, 1985; Ho and Weisblat, 1987). Glial processes are juxtaposed to sites of neurogenesis in the leech germinal plate, suggesting a role in glial guidance of neuronal outgrowth and migration (Cole et al., 1989).

The leech glia have proved ideal for microelectrode studies, and have contributed to our understanding of glial electrophysiology, pH and potassium regulation, and sensitivity to neurotransmitters (see Chapters 11 to 20).

The Annelid Blood-Central Nervous System Interface Is Leaky to Small Ions. The leech ganglia and connectives lie in a blood sinus, separated from the blood by an "endothelium" and basal lamina. Kuffler and Potter (1964) found that (protein-bound) trypan blue was excluded by the endothelium. However, by using the membrane potential of the leech neuron as an indicator of its own local ionic microenvironment, Nicholls and Kuffler (1964) showed that there was relatively rapid exchange between the medium and the neuronal surface (half-time for exchange of potassium and sucrose for sodium < 10 seconds) *in situ*. Together with electron microscopic evidence showing absence of tight junctions in the cell layers lining the blood sinus, these studies showed that the leech ganglion has no surface barrier to small molecules. Kai-Kai and Pentreath (1981) showed that ionic lanthanum has free access to the leech ganglionic interstitium. Studies on the connective gave similar results. Ion substitution and electron microscopic studies in another annelid, the serpulid worm *Mercierella* show that this species also lacks a blood-brain barrier (Skaer et al., 1978); this appears to be the general annelid pattern.

The Extracellular Space, Not Glia, Is the Major Route for Exchange of Materials Within the Nervous System. As the leech glial membrane potential did not change during substitution of sucrose for sodium (Nicholls and Kuffler, 1964), it was concluded that these molecules moved through the extracellular space. This was also the conclusion from studies with radiolabeled sucrose, inulin, and dextran (Nicholls and Wolfe, 1967). These experiments led to the modern view that the extracellular space is the most important medium for exchange of materials within the nervous system (see Chapter 26), and between blood and neurons, although there may be situations where glial cells provide local trophic support to nerve cells (Wolfe and Nicholls, 1967; see also the sections *Lower Mollusks* and *Cephalopod Mollusks* below), or act as a pathway for redistribution of ions (see Chapter 37).

Arthropods

Glial Cells in Insects and Crustacea Are Classified by Morphology and Location. There are sufficient homologies of structure and function among arthropods (Xiphosurans such as the horseshoe crab *Limulus,* arachnids such as spiders and scorpions, Crustacea, and insects) to regard them as closely related animal groups (Figure 6-1), although details of their evolutionary relationships are not fully understood (Barnes, 1980).

The nervous systems of insects and Crustacea have been the subject of intense morphological and physiological study, and several attempts at glial classification have been made. Strausfeld (1976) illustrates a range of insect glial morphologies from studies since Ramón y Cajal's early work (Figure 6-3). The most coherent descriptions come from detailed examination of individual species, in which physiological and anatomical studies have been done in parallel [e.g., insects: cockroach (*Periplaneta*), fly (*Musca*), bloodsucking bug (*Rhodnius*), fruit fly (*Drosophila*), stick insect (*Carausius*); Crustacea: crab (*Carcinus*), crayfish (*Procambarus*)].

This is a generalized summary, based on insects and higher Crustacea. An outer layer of glial cells, the *perineurium* forms a complete sheath around ganglia and interganglionic "connectives" (Figure 6-4) and, to a variable extent, around peripheral nerves (Lane, 1974; Lane and Abbott, 1975; Treherne and Schofield, 1981). Small neurons and axons are surrounded by glial processes, either singly or in bundles. Giant axons are enveloped in a multilayered wrapping formed by multiple turns of *mesaxonal* glia. Gap junctions between the insect perineurium and the underlying glia give rise to cell-cell

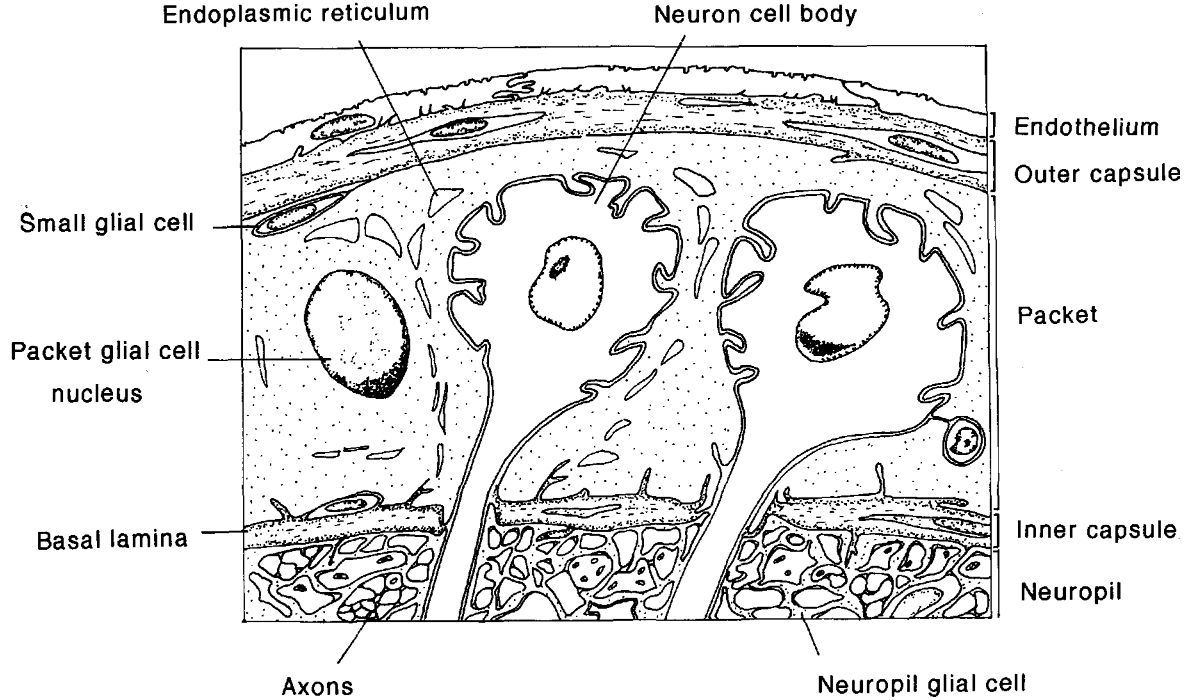


FIG. 6-2. The three main types of glial cell in the leech ganglion (Hirudinea, Annelida): *1*, Small glial cells under the outer capsule sheath, 2, packet glial cell surrounding neuron somata, and 3, neuropil glial cell around small axons. Both the endothelium and glial layers are permeable to small ions. [From Coggeshall and Fawcett (1964), with permission.]

electrical coupling. Insect perineurial cells are metabolically active (Wigglesworth, 1960) and show many morphological features of transporting epithelia (Maddrell and Treherne, 1967), including high mitochondrial density, elaboration of junctional complexes, presence of fat globules, and clusters of glycogen granules. The glial cells surrounding large neuron cell bodies send "trophospongial" processes into the perikarya and axon hillock regions (reviewed in Lane, 1981; Sandeman and Luff, 1973). The long cellular processes of insect glial cells may contain dense arrays of microtubules (Lane, 1974). In some insects, parts of the interstitial space between the glial cells are expanded into *lacunae* (the *glial lacunar system*) filled with dense extracellular material containing anionic substances such as hyaluronic acid and glycoproteins that may act as an ion reservoir (Treherne et al., 1982).

More detailed numerical classification of glial subtypes (type-1, type-2, etc.) has been attempted for *Rhodnius* and *Periplaneta* (Wigglesworth, 1959, 1960), and *Musca* (Sohal et al., 1972; Strausfeld, 1976), but these classifications are based on somewhat arbitrary morphological criteria (position, relation to neurons), and become confusing in any comparative account because the numbering systems do not correspond. A numerical classification is avoided here.

The main difference between insects and decapod Crustacea (crayfish, crab, lobster) is that the crustacean groups have a system of vascular channels running within the nervous system (Abbott, 1971a, 1971b) (Figure 6-5). Invaginations of the perineurium form the layer of perivascular glia lining the blood channels.

Glia of the Insect Embryo Act as Guideposts in Development. The insect embryo has several functionally distinct classes of glia, some with important roles in development (Jacobs and Goodman, 1989; Jacobs et al., 1989). Ablation of one particular glial cell in the grasshopper, the segment boundary cell, causes disturbance of neuronal growth cone guidance in development (Bastiani and Goodman, 1986). In *Drosophila,* two pairs of midline glia, the MGA and MGM cells, are important in the formation and separation of commissural axon tracts across the midline, as shown by abnormalities of the commissures in mutants involving these glia (Klämbt and Goodman, 1991).

Glial Cells of the Arthropod Perineurium Contribute to the Blood-Brain Barrier. Insects, higher arachnids, and decapod Crustacea, possess a blood-brain barrier (reviewed in Abbott et al., 1986b). It has been proposed that a blood-brain barrier is necessary

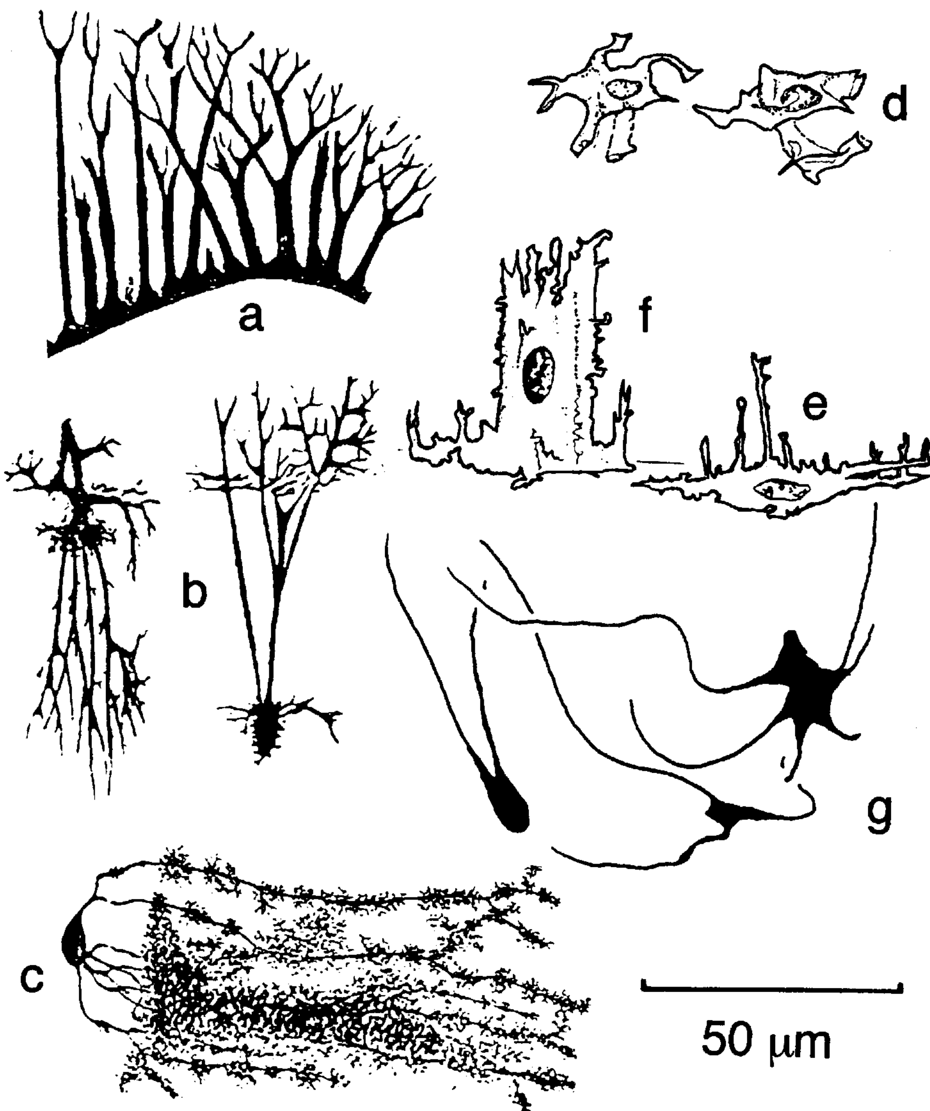


FIG. 6-3. Forms of insect neuroglia. [From Strausfeld (1976), with permission.] (A) Epithelial cells. (B) Displaced epithelial cells. (C) Radiate glial cells. (D) Satellite glia of the lamina cell-body layer. (E) Marginal glia beneath the inner face of the lamina. (F) Epithelial cell of the laminal neuropil. (G) Multipolar glia of the antennal lobes. [Figures A, B, and C from Ramón y Cajal and Sanchez (1915); Figure G from Scharrer (1939).]

where the nervous system is involved in complex sensory and motor processing because of the need for ionic homeostasis around central integrating synapses (Abbott et al., 1986a). Some insects have additional requirements for an effective barrier, since their blood (hemolymph) may contain unusually high or fluctuating levels of potassium (Lettau et al., 1977).

Unlike the situation in higher vertebrates, where the blood-brain barrier is formed by endothelial cells lining cerebral microvessels, the barrier in insects and Crustacea is formed by glial cells. In insects the barrier resides in the innermost zone of the perineurium, or "bracelet cell" layer (Treherne and Pichon, 1972; Lane, 1991). Extracellular tracers such as horseradish peroxidase, microperoxidase, and lanthanum fail to penetrate into the interstitial space around neurons. The barrier has been attributed to tight junctions in the perineurium (Lane, 1991), but these have not been seen in all insects; septate junctions (Juang and Carlson, 1992) or extracellular material in narrow interstitial clefts may contribute to the restriction in some cases.

The perineurial barrier is relatively tight to small ions (reviewed in Treherne and Schofield, 1981; Schofield and Treherne, 1984, Schofield et al., 1984), and may be under hormonal modulation, as the stress hormone octopamine causes a decrease in potassium permeability (Schofield and Treherne, 1986). This suggests that in periods of stress, the brain would be more protected from fluctuations in plasma composition and hence better able to produce coordinated neural activity. No equivalent tightening mechanisms have been described for the vertebrate endothelial barrier.

The situation in insect peripheral nerves is variable; large nerves generally have a perineurial barrier, but in small nerves and nerve terminals the bar-

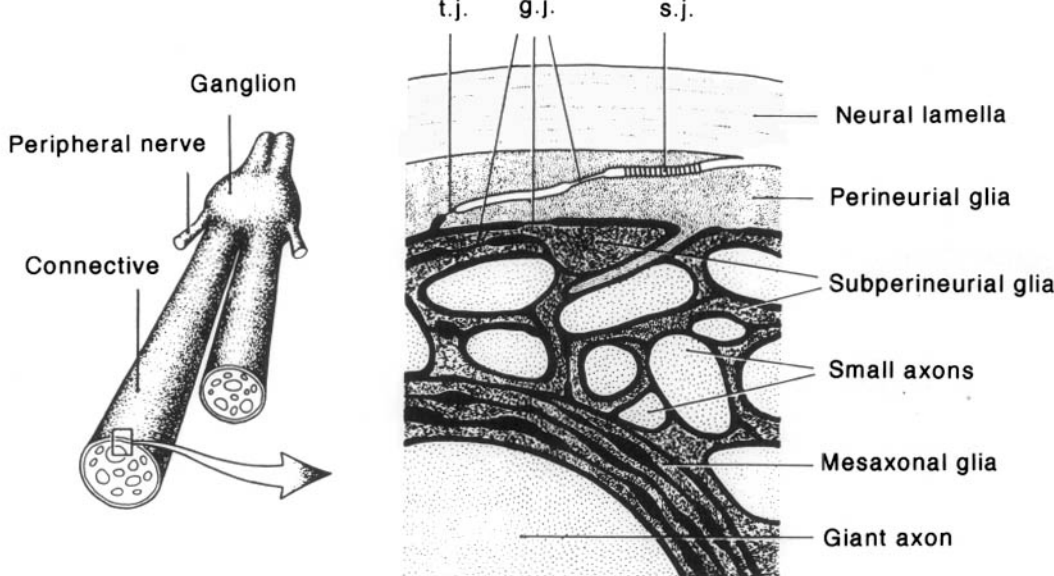


FIG. 6-4. Outer layers of the cockroach *Periplaneta* (Insecta) central nervous system connective, to show the three main types of glia: perineurial, subperineurial, and mesaxonal. The tight junctions (*t.j.*) of the perineurium form a diffusion barrier at the blood-nervous tissue interface. Gap junctions (*g.j.*) and septate junctions (*s.j.*) are also present. [From Treherne and Schofield (1982), with permission.]

rier may be leaky to extracellular tracers (Lane, 1991). Neurosecretory zones have a permeable interface between the nerve terminals and the blood (Lane et al., 1975).

In decapod Crustacea the barrier is formed by the glial perineurium surrounding the ganglia, and by its invaginations the perivascular glia, relatively tight to ionic lanthanum (Lane et al., 1977). Freeze fracture information is lacking, but thin sections show occasional tight junctions (Lane and Abbott, 1975). Electrophysiological studies show that the crustacean perineurium is leakier to potassium than that

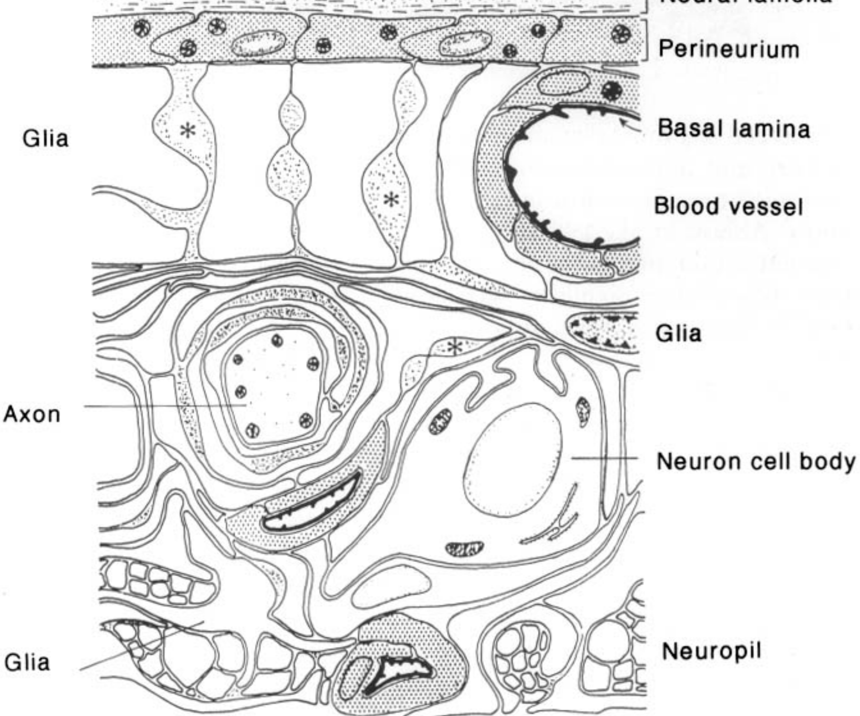


FIG. 6-5. Glial cells of the cerebral ganglion of the shore crab *Carcinus* (Crustacea). Extensions of the glial perineurium (shaded) form perivascular cuffs. Other glia with distinct morphologies can be seen under the perineurium (*subperineurial*), surrounding the cell body zone (*sheathing*), winding around axons (*mesaxonal*), enclosing neuronal somata, with trophospongial invaginations (*perineuronal*), and surrounding bundles of small axons in the neuropil (*neuropilar*). The perineurial and perivascular glia form the blood-brain barrier. [From Abbott (1971a), with permission.]

of insects (Abbott and Pichon, 1987; Butt, 1991). Electron microscopic studies show that the peripheral nerves of decapod Crustacea have a relatively thin and incomplete perineurium, and electrophysiological studies confirm that the peripheral nerve sheath in crab and crayfish is relatively leaky to ions (Abbott et al., 1975; Abbott and Pichon, 1987). In lobster nerve roots, the giant axons are somewhat more protected; here the blood-nerve barrier appears to be a combination of incomplete tight junctional zones, a tortuous diffusion pathway produced by the multilamellar glial ensheathment, and diffusional restriction caused by extracellular material in the cleft (Villegas and Sanchez, 1991).

The relatively open blood-nerve barrier in Crustacea compared with insects may reflect the fact that crustacean blood is a more suitable (and stable) ionic medium for neuronal function than insect blood. The retention of a barrier around ganglia, connectives and giant axons may reflect the need for better ionic homeostasis around integrating synapses and axons involved in survival behaviour such as fast escape responses and prey catching.

Glia at the Arthropod Neuromuscular Junction Are Specialized for Transmitter Uptake. Glial cells are closely apposed to the nerve terminals at the neuromuscular junction of both excitatory (glutamatergic) and inhibitory (GABAergic) nerves in arthropods. Autoradiographic evidence shows that here glial cells are the predominant site of transmitter uptake, an important step in transmitter inactivation and recycling (Faeder and Salpeter, 1970; Orkand and Kravitz, 1971; Evans, 1974; Horwitz and Orkand, 1980; see Chapter 15, this volume).

Arthropod Glial Anatomy Contributes to Regulation of the Neuronal Ionic Microenvironment. A prominent feature of glial cells in crustacean (but not insect) central nervous system connectives is a system of transcellular tubules called the *tubular lattice,* which is accessible to extracellular tracers (Lane and Abbott, 1975; Lane et al., 1977). The rapid clearance of potassium away from the axon following repetitive activity has been attributed to this tubular system (Shrager et al., 1983). In the retina of the honeybee drone, uptake of potassium and chloride by the glial cells helps to regulate the neuronal microenvironment during periods of photoreceptor stimulation (reviewed in Coles, 1989; Coles and Schneider-Picard, 1989; Coles et al., 1989).

Axon-Glial Anatomy in Crustacea Permits Chemical Signaling from Axon to Glial Cell. The giant axons of squid are closely invested by a glial (Schwann) cell sheath, and a neurotransmitter cascade is involved in axon-Schwann cell signaling (see below). A similar geometry around crustacean giant axons within the central nervous system connectives proves also to permit axon-glial signaling (Smiley and Lieberman, 1980; Lieberman et al., 1981). These studies have helped to establish the generality of chemical signaling between axons and their surrounding glial cells, recently extended to vertebrates (see Chapter 31).

Lower Mollusks

Lower Molluskan Glia Have a Variety of Morphologies and Associations with Neurons. In lower mollusks, the neural ganglia follow a common pattern, with neuronal cell bodies clustered at the periphery and the synaptic neuropil concentrated at the core. There are no vascular networks within the ganglion, but blood spaces may occur around the cell bodies. As in insects and Crustacea, glial types are mainly identified according to location and morphology, with "plasmatic" or "protoplasmic glia" mainly at the periphery, and "fibrous glia" mainly associated with axonal tracts (Radojcic and Pentreath, 1979). There is no clear equivalent of the *perineurium* of arthropods. Glia associated with large-neuron somata invaginate as trophospongium. In bivalves such as the freshwater mussel *Anodonta,* there are very few glial cells interspersed between axons (Treherne et al., 1969; Lane and Treherne, 1972) (Figure 6-6).

Lower Mollusks Lack a Blood-Central Nervous System Barrier. Electron-dense tracers have been used to show that the blood-central nervous system interface is leaky in the bivalve *Anodonta* (Treherne et al., 1969; Lane and Treherne, 1972; Sattelle and Howes, 1975) and in several gastropods (Mirolli and Crayton, 1968; Pentreath and Cottrell, 1970; Reinecke, 1975, 1976). Studies with lanthanum are complicated by the fact that the charged tracer appears to be excluded from (charged) fibrillar zones within the extracellular space. Ion-substitution experiments in freshwater gastropods and bivalve nervous systems confirm that the interface is relatively open (Sattelle, 1973), but show that in some cases neuronal function can continue in a low-sodium medium, possibly supported by a local glial sodium store (Treherne et al., 1969) (Figure 6-6). An analogous calcium ion store has been proposed in the marine mollusk *Aplysia* (Keicher et al., 1992).

Anatomical Relations Underlie Neuron-Glial Interaction in Molluskan and Annelid Ganglia. The close glial ensheathment of neurons, with trophospongial

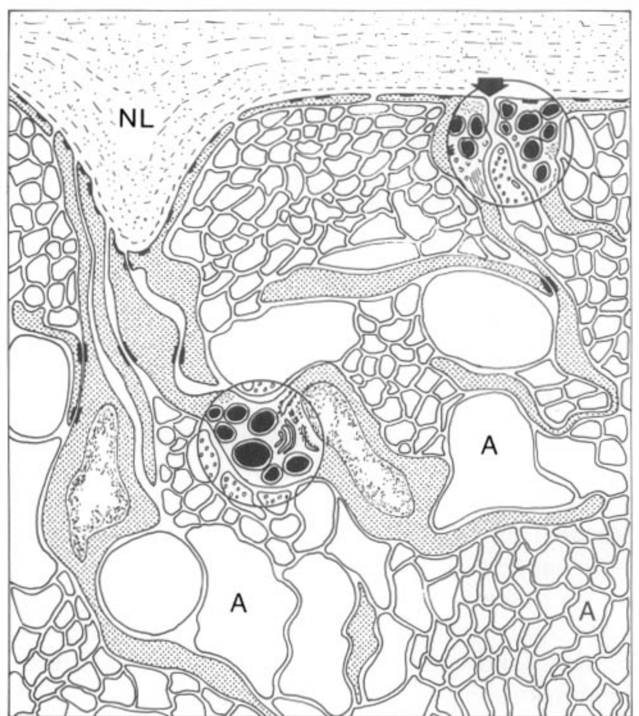


FIG. 6-6. Glial cells (*shaded*) and axons (*A*, *unshaded*) of the cerebrovisceral connectives of the central nervous system in the freshwater mussel, *Anodonta cygnea* (Lamellibranchiata, Mollusca). The circles highlight fine-structural detail. Note the lack of junctional specializations between the cells of the outer glial sheath (*arrow*, absence of diffusion barrier), and presence of dark granules in glial associated with the larger axons, that may represent an intracellular sodium store. The dark bars are desmosomes and hemidesmosomes providing mechanical coupling between glia cells, and between glia and neural lamella (*NL*) respectively. [Based on Treherne et al. (1969), with permission.]

invaginations, permits a range of short-range interactions between neurons and glia. Autoradiographic techniques have provided evidence for trophic support of neurons by glia, and for modulation of this support by signals liberated by neurons (Pentreath, 1982; Pentreath and Pennington, 1987).

Cephalopod Mollusks

Cephalopod Glial Cells Have Complex Morphologies. Coleoid cephalopods include squid, octopus, and cuttlefish, marine mollusks with highly developed nervous systems able to compete successfully with other marine predators, such as fish. Cephalopods are the only invertebrate group with a completely closed vascular system, formed by a continuous vessel network within the tissues.

The morphologies of cephalopod glial subtypes show many similarities with those of higher vertebrates. Lenhossèk (1896) described "bushy glial cells" in the plexiform zone of the optic lobes of cephalopods, the first clear demonstration of branching neuroglia in any invertebrate. Ramón y Cajal (1917) who had done so much to demonstrate the glial types in mammalian brain, confirmed the plexiform zone glia of cephalopods, and also described "epithelial cells" surrounding neurons in the outer granule layer of the optic lobe and "protoplasmic astrocytelike cells" throughout the lobe. Cells like fibrous astrocytes were seen by Gariaeff (1909) around neuronal perikarya and invaginating them as trophospongium. Young (1971) applied many of the classical gold and silver staining techniques of Golgi, Ramón y Cajal, and later histologists to the octopus brain, and succeeded in a very detailed mapping of the neurons and their connections; under some conditions the stains showed glial cells rather clearly. Classes of *protoplasmic glia* associated with neurons and *fibrous glia* associated mainly with axon tracts could be distinguished. Cardone and Roots (1990) showed glial fibrillary acidic protein (GFAP) immunoreactivity in both octopus and snail brain.

Certain Glial Cells Are Associated with Cephalopod Blood Vessels and the Glio-vascular System. Bogoraze and Cazal (1944) were the first to describe a close association between one class of cephalopod glial cell and the blood vessels. Stephens and Young (1969) confirmed the association between glia and vessels, and described a "gliovascular" system of glial-lined spaces radiating from the perivascular space deep into the octopus brain. Electron microscopy of the gliovascular system (E. Gray, 1969) showed that it contained collagen, smooth muscle cells, and fibrocytes, suggesting it might play a dynamic role in the movement of interstitial fluid. Grapelike clusters of "lymphoid" spaces around venous channels (Stephens and Young, 1969) could act as the drainage route by which fluid returned to the venous system. In the cuttlefish *Sepia officinalis*, the gliovascular system is not prominent in the vertical and optic lobes, although there are expansions of the extracellular space, particularly around arteries (Bundgaard and Abbott, 1992). However, there is good radiotracer evidence for a flowing interstitial fluid in *Sepia* (Abbott et al., 1985), suggesting the interstitial spaces present are sufficient to sustain the flow.

There are some instructive parallels with the mammalian brain. The vertebrate central nervous system lacks the lymphatic vessels found in other tissues, and it was originally assumed that fluid drainage was entirely via cerebrospinal fluid into arachnoid villi and hence into cerebral veins. However, it is

now clear that perivascular spaces may act as low-resistance pathways for interstitial fluid flow in mammalian brain, and that up to 50% of the interstitial fluid and cerebrospinal fluid may leave the brain via this route, connecting to the cranial nerves and lymphatics of the neck (Bradbury and Cserr, 1985; Ichimura et al., 1991). A system of glial-lined spaces filled with flowing interstitial fluid may then be a feature of both invertebrate and vertebrate brain; a flowing interstitial fluid permits more effective ionic homeostasis, distribution of neuroactive compounds (e.g., modulator peptides), and removal of wastes than does a stagnant system (Abbott et al., 1985).

Glial Cells Contribute to the Cephalopod Blood-Brain Barrier. Early electron microscopic studies by Barber and Graziadei (1965, 1967) identified two classes of cell forming the wall of octopus cerebral blood vessels, endothelial cells, and pericytes, but as neither layer was invariably complete, they concluded there was no blood-brain barrier. More rigorous study of the optic and vertical lobes in the cuttlefish *Sepia* showed that a continuous (and in many cases "seamless") layer of perivascular glial endfeet was always interposed between the blood and the neurons, except in the case of neurosecretory cells (Bundgaard and Abbott, 1992). Studies with electron-dense tracers horseradish peroxidase and lanthanum showed that these molecules met diffusional restriction at the glial level (Abbott and Bundgaard, 1992). Thus in the microvessels, glial cells form the blood-brain barrier of the cuttlefish (Figure 6-7). Studies on the squid and octopus confirm the same pattern (Bundgaard et al., 1994), which therefore is likely to apply to all coleoid cephalopods. In arteriolar vessels the diffusional restriction is formed by the (mesodermal) pericyte layer (Abbott and Bundgaard, 1992). The barrier in cephalopods appears not to be due to conventional tight junctions (*zonulae occludentes*), but rather is achieved with little narrowing of the intercellular cleft. Periodic electron-lucent "linker" structures spanning the cleft, and a dense extracellular matrix may together be responsible for the diffusional restriction, a novel type of occluding junction (Abbott et al., 1992a; Bundgaard et al., 1994). The axons connecting the *Sepia* optic lobe to the retina are accessible to horseradish peroxidase and hence lack a barrier (Abbott et al., 1986b).

The Cephalopod Blood-Brain Barrier Gives Insights into the Evolution of the Vertebrate Blood-Brain Barrier. The presence of a blood-brain barrier in insects, Crustacea, and cephalopod mollusks—but the lack of a barrier in lower invertebrates—led to the suggestion that a barrier is needed for the more complex kinds of integrative and analytical activity of the nervous system (Abbott et al., 1986a). It was proposed that the evolutionary pressure to develop a barrier came from the need to preserve ionic homeostasis around the synaptic zones responsible for central integration, given the vulnerability of "graded" synaptic potentials to fluctuations in the ionic composition. This idea is strengthened by the observation

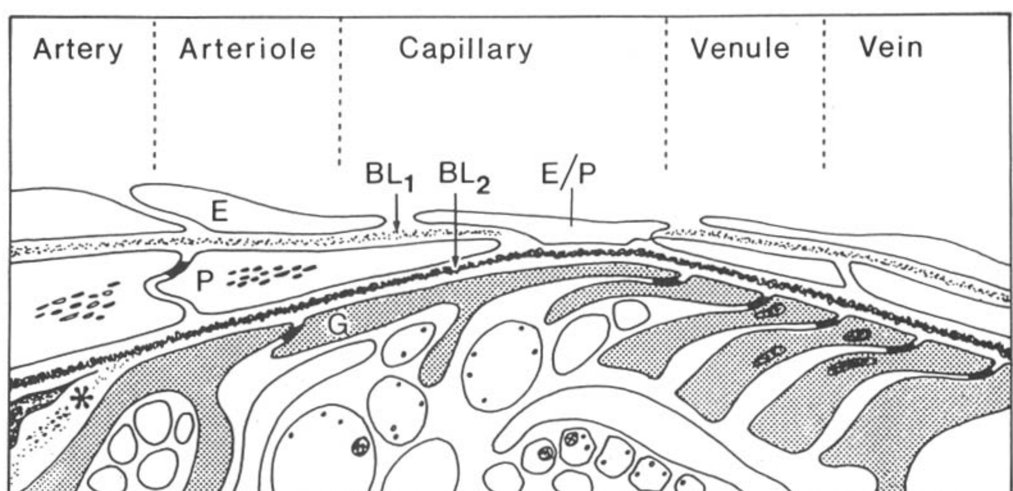


FIG. 6-7. The blood-brain interface in the cuttlefish *Sepia officinalis* (Cephalopoda, Mollusca). Schematic diagram (not to scale) from the arterial to venous end of the microvasculature, based on electron microscopy of the vertical and optic lobes of the brain. *E*, endothelial cell; *P*, pericyte; BL_1 and BL_2, basal laminae; *G* (*shaded*), perivascular glial layer; *asterisk*, collagen-filled extracellular space. In the smallest vessels, only a single class of endothelial/pericyte (*E/P*) can be seen. Black bars between cells show occluding juctions. The blood-brain barrier is formed by glia in the capillaries and venules, and by pericytes in arterioles. [From Abbott and Bundgaard (1992), with permission.]

that axons, conducting "all-or-none" action potentials do not need such fine control, as shown by the absence of a blood-nerve barrier in crustacean, cephalopod, and small insect nerves.

The presence of a glial barrier in invertebrates and a primitive vertebrate group the elasmobranchs (see below) together with the presence of glial-ependymal barrier in higher vertebrate brains in early embryonic stages suggests that a glial barrier is the primitive condition, and an endothelial barrier as found in higher vertebrates, including mammals, is a later evolutionary feature. The pericyte layer of cephalopod arterioles may then represent an intermediate transition stage (Abbott, 1992). The "linker junction" of the cephalopod blood-brain barrier also shows some features seen in glial junctions of the embryonic vertebrate neural tube (Møllgård et al., 1987), and in the wider parts of the junctional zones in mammalian endothelia (Firth et al., 1983). Thus the location and fine structure of the cephalopod barrier junction can give useful insights into the evolution of occluding junctions in general, and the blood-brain barrier in particular (Abbott, 1991, 1992).

Schwann Cells Associated with the Squid Giant Axon Are Involved in Axon-Schwann Cell Signaling and Coordination. In most vertebrates, the Schwann (glial) cells around peripheral axons are too small for physiological study *in situ,* so that most information comes from dissociated and cultured cells (see Chapter 51). The Schwann cells of the giant axon of the squid are relatively accessible and large (cell bodies around $70 \times 20 \times 1$ μm), with elongated processes running along the axonal axis (Brown and Abbott, 1993). They have a prominent system of transcellular tubules (glial tubular system) like the tubular lattice of crustacean adaxonal glia (Zwahlen et al., 1988; Brown and Abbott, 1993) and the intercellular cleft has a restriction at the axonal end where a single strand of intramembranous particals is seen in freeze-fracture replicas (Zwahlen et al., 1988). Electron-dense tracers in the bathing medium are able to penetrate the glial tubular system and reach the periaxonal space (Brown and Abbott, 1993). Morphometric studies show that the tubular system is likely to be the predominant route for extracellular ion flux (Zwahlen et al., 1988; Brown and Abbott, 1993).

The size of the squid Schwann cells and axons has permitted microelectrode recording and pharmacological and biochemical study. There is evidence for a "transmitter cascade" of chemical signaling from axon to Schwann cell (Villegas, 1984; Lieberman et al., 1989; Evans et al., 1991), capable of switching the Schwann cell from a quiescent to an activated state following axonal activity (see Chapter 31). There is also evidence for a trophic role of Schwann cells on the giant axons, specifically in the transfer of proteins (Lasek and Tytell, 1981; Buchheit and Tytell, 1992).

NONMAMMALIAN VERTEBRATE GLIAL CELLS

Evolution of Glial Structure and Function

Figure 6-8 shows a vertebrate evolutionary tree. The morphology and function of glial cells in lower vertebrates can give clues to glial evolution, and help to explain the origin of patterns seen in higher groups. The vertebrate neural tube is derived from ectoderm, and in many lower vertebrates, the epithelial nature of glia remains prominent. In the primitive neural tube, glial cells or their precursors span from pial to ventricular surface, forming the radial glia (see Chapter 4). Cells with processes extending back from the ependyma but not attached to the pia are termed ependymal tanycytes. In mammals and birds, radial glia are a transient population forming a scaffold for migration of neuroblasts to their final locations, and largely transformed into astrocytes in the adult (Voigt, 1989; Misson et al., 1991). Radial glia and tanycytes are retained into the adult in many lower groups (reviewed in Roots, 1986), along with other glial epithelial features: barrier-forming

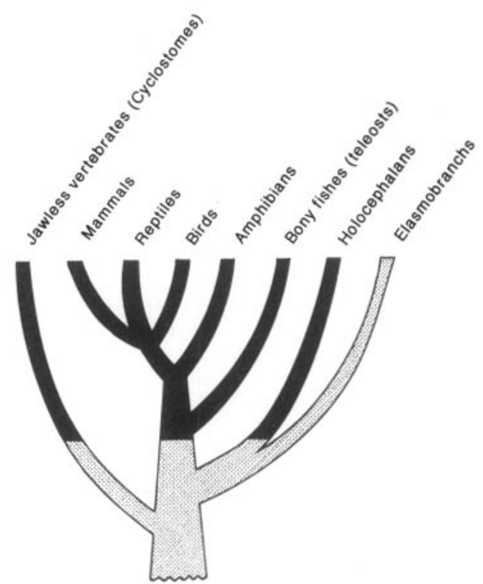


FIG. 6-8. Evolutionary tree of vertebrates; the base is approximately 500 million years ago, the tips of the branches represent present time. *Stippled area*, glial blood-brain barrier; *black area*, endothelial barrier. [From Abbott, (1992), based on Cserr and Bundgaard (1984), with permission.]

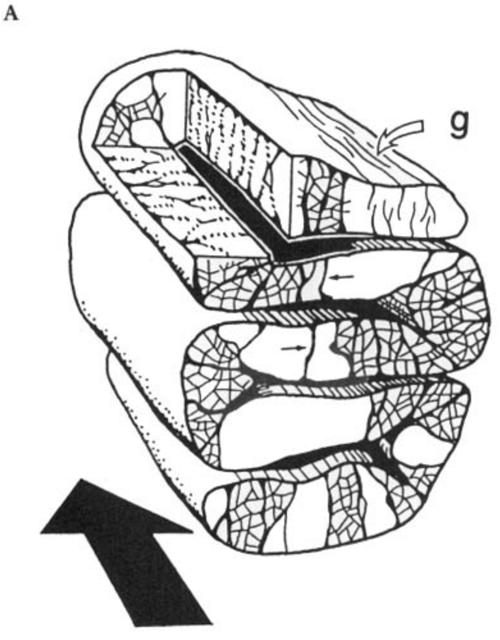
potential, desmosomes, and intermediate filaments closely related to those of the skin. The relations between hematogenous cells and glia in teleost fish highlight some important features of the immune system in the brains of higher groups.

Fish: Cyclostomes, Teleosts, and Elasmobranchs

Cyclostomes Have Epithelioid Glial Cells. Cyclostomes, the class of Agnatha that includes the lamprey and hagfish, diverged from mainstream jawed vertebrates in pre-Carboniferous times (>300 MYr ago). Their central nervous system shows several primitive features, including the retention of Rohon Beard cells in the adult (reviewed in Scholes, 1991; Scholes et al., 1992). They have almost no myelin, and, correspondingly, few oligodendrocytes. The neural tissue is vascularized chiefly at its surface, with very few penetrating vessels; they are absent in spinal cord. The astroglia have a similar radial form throughout the central nervous system, linked by desmosomes and full of intermediate filaments, which are not immunoreactive for GFAP (Dahl and Bignami, 1973; Dahl et al., 1985).

The Teleost Optic Nerve Retains Primitive Features to Cope with Physical Stress. In the embryology of the vertebrate optic nerve, the germinal (ependymal) zone is lost as a result of the "sculpting" process of selective cell death. The avascular optic nerves of bony fish (teleosts) contain a single class of unusual "reticular" astrocytes, which retain their radial orientation, but now connect pial surfaces and form a series of lacelike partitions through which the axons run (Maggs and Scholes, 1990) (Figure 6-9). The movements of the eyes impose large stresses on the teleost optic nerve, and several anatomical specializations result. Nerve axons display a zigzag pattern, allowing length changes, and the reticular astrocytes provide a scaffold, maintaining the organization, and possibly imposing periodicity on nodal distribution (Scholes, 1991). The reticular astrocytes also segregate axons in the left and right optic tracts, leading to the completely crossed optic chiasm unique to fish (Scholes et al., 1992). The astrocytes are linked by desmosomes and contain cytokeratins (Giordano et al., 1989; Druger et al., 1992) rather than the GFAP filaments characteristic of higher groups, and in this they resemble both the floor plate cells of the developing amphibian neural tube

A



B

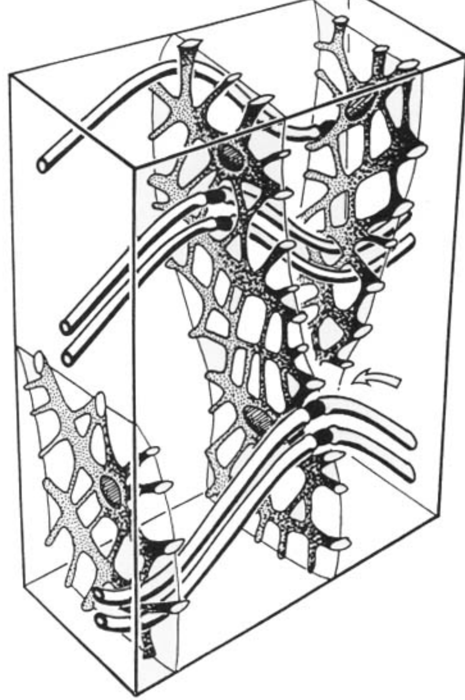


FIG. 6-9. **(A)** Arrangement of the reticular astrocyte network in the ribbon optic nerve of the cichlid teleost fish. The *large black arrow* shows the direction of the optic fibers. *g.* indicates the germinal margin of the ribbon, in which preastrocytic cells have fine lamellar processes running longitudinally as indicated, through distances equivalent to several partition repeats. **(B)** The relations between reticular partitions (*stippled*), the nerve fiber waves, and the nodes of Ranvier (*arrow*) in the chichlid optic nerve. The oval structures (hatched) are astrocyte nuclei. [From Scholes et al. (1992), with permission.]

(Holder et al., 1990) and the cells of the skin. Retaining a more epithelioid phenotype in the fish optic nerve glia allows the nerve to preserve elasticity and resistance to stretch, no longer needed in the brain enclosed in a bony skull. Interestingly, GFAP expression increases in the optic nerve after injury (Stafford et al., 1990), suggesting that reactive glia can recruit from a phenotype closer to that of the brain.

Fish Myelin Holds Clues to Myelin Evolution. Skate (elasmobranch) and teleost brains contain myelin proteins with close similarities to P_0, the myelin protein found only in the peripheral nervous system of higher vertebrates (Jeserich and Waehneldt, 1987; Jeserich et al., 1990; reviewed by Kirschner and Blaurock, 1992). Fish thus mark the transition from P_0 to proteolipid protein (PLP) as the major myelin protein component (see Chapter 38).

Teleost Brain Contains Large Numbers of Glial-Associated Hematogenous Cells. The teleost central nervous system has three main classes of glial cell, oligodendrocytes, ependymal tanycytes, and radial glia (Wolburg and Bouzehouane, 1986; Sivron et al., 1992). Fish have a relatively weak immune system, having not yet developed the germinal zones for hemopoetic tissue in bone marrow and lymph nodes, nor the ability to make high-affinity antibodies as in higher vertebrates (Du Pasquier, 1992). In mammalian brain, cells derived from blood (e.g., lymphocytes, macrophages) are present in small numbers, generally associated with sites of inflammation. By contrast, hematogenous cells are numerous in teleost brain, and are found associated with glia, not neurons (Dowding and Scholes, 1993). The associations are particularly clear in the optic tectum, which has a marked vertical layering segregating white and gray matter and other zones (Figure 6-10). Macro-

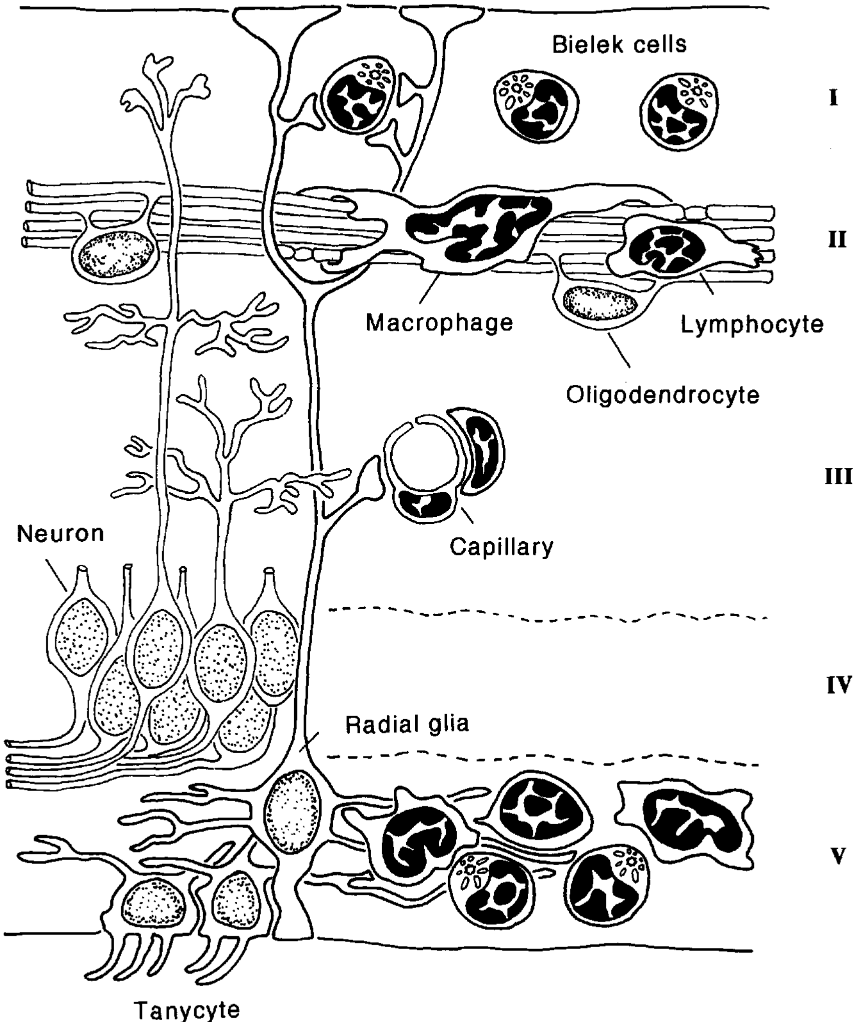


FIG. 6-10. Cell types in the teleost optic tectum. Fish lack the regular networks of ramified microglial cells found in the central nervous system in mammals. Instead, varied hematogenous forms of leukocytes are present in large numbers in the brain and spinal cord, associated with glia not neurons. This diagram shows their localization determined immunocytochemically and by electron microscopy (Dowding and Scholes, 1993) in the optic tectum, whose orderly vertical layering displays the specificity of these associations.

The leukocytes are clustered in three of the five strata (I–V) of the tectum, as follows: *I*, the *stratum marginale*, contains mainly Bielek cells, a special category of leukocyte found in the hematogenous organs and central nervous system in fish. Here they are ringed by endfeet of the distal processes of the radial glia, the *stratum opticum*, composed of dense fascicles of myelinated afferent fibres. Numerous macrophages and lymphocytes are specifically associated with the myelin here and elsewhere in the central nervous system: in spinal cord white matter they outnumber the oligodendrocytes. *V*, the *subependyma*, a glial plexus formed by radial glia and tanycytes which contains numerous leukocytes of different types, mainly lymphocytes and Bielek cells. By contrast, all types of leukocytes are rare in the neuropil and nerve cell layers of the tectum (strata III and IV). The association of radial glial endfeet with capillaries is shown. [By permission of J Scholes, unpublished data.]

phages congregate in white matter, where they remove myelin debris. A class of leukocyte, the Bielek cell, is strongly associated with glial end feet in the marginal zone (strium marginale) and subependymal plate, an association that may be designed to prevent their free passage into the neuronal zones, or to facilitate immune interactions. The subependymal plate also contains large numbers of lymphocytes and macrophages.

The Teleost Central Nervous System Has Strong Regenerative Capacity. The fish central nervous system has a strong capacity for regeneration and repair (Attardi and Sperry, 1963; Schwartz et al., 1985; Battisti et al., 1992), possibly associated with the numbers and efficiency of hematogenous macrophages. The system may only work well because fish have not evolved mechanisms for generating high-affinity antibodies. The relative segregation of the higher vertebrate nervous system from hematogenous cells, with myelin housekeeping passed over to microglia and astrocytes, may give the brain of higher vertebrates greater protection from autoimmune attack, but at the expense of the loss of repair capacity (Dowding and Scholes, 1993).

Elasmobranchs Preserve Many Primitive Evolutionary Features. Elasmobranchs are the dominant group of extant cartilaginous fish (Class Chondrichthyes). They preserve several primitive features including the anatomy of the heart and gill arches, and aspects of digestive and autonomic physiology, although modern elasmobranchs show a great range of "advanced" and "primitive" characteristics. The brain is also relatively simple in structure, with a straightforward linear organization of prosencephalon (telencephalon and diencephalon), mesencephalon, and rhombencephalon, and an absence of surface convolutions over the cortex and cerebellum (Smeets et al., 1983). Tanycytes are a prominent class of elasmobranch glia found throughout the central nervous system (Horstmann, 1954).

The Elasmobranch Blood-Brain Barrier Is Formed by Perivascular Glial Cells. In the major vertebrate groups, the blood-brain barrier is formed by endothelial cells (Figure 6-8, see below). However, elasmobranchs resemble the invertebrate pattern, in having a glial blood-brain barrier (Brightman et al., 1971; Bundgaard and Cserr, 1981) (Figure 6-8). The endothelial junctions are leaky to horseradish peroxidase and show interrupted strands of intramembranous particles in freeze-fracture replicas, while the endfeet of perivascular glial cells are coupled by tight junctions (Figure 6-11) that exclude horseradish peroxidase and show dense networks of intramembranous particles (Gotow and Hashimoto, 1984). The glia limitans under the pial surface is also coupled by tight junctions, so that the brain is effectively sealed in behind a wall of glial cells on both its parenchymal and its pial surfaces.

In most vertebrates a glioependymal barrier is present in the developing neural tube (Møllgård et al., 1987), but disappears later in development. It is intriguing that in glial morphology, as with some other tissue characteristics, elasmobranchs preserve embryonic vertebrate features, adding weight to the idea that in certain situations "ontogeny repeats phylogeny" (Abbott, 1991, 1992). Young (1950) has argued that elasmobranchs have been so successful as large marine predators that they have not been subjected to the same evolutionary pressures as other groups, which may explain the retention of the primitive features. The ratfish *Chimaera monstrosa*, representative of the other main group of cartilaginous fish, the Holocephali, has an endothelial blood-brain barrier (Bundgaard, 1982; Cserr and Bundgaard, 1984) (Figure 6-8).

The Blood-Brain Barrier Glia of Elasmobranchs Retain Glial Properties While Controlling Blood-Brain Exchange. This volume documents the ways in which glial cells help to maintain a proper environment for neural development and function. It is of interest to establish whether the blood-brain barrier glia of elasmobranchs share these properties, or whether they have had to abandon their glial characteristics in order to function as a barrier controlling blood-brain exchange. Radiotracer methods have been developed for studying the permeability and transport of the mammalian blood-brain barrier, and can be easily modified for elasmobranchs (Abbott et al., 1988a). It is found that the glial barrier expresses glucose and leucine transporters (as does the mammalian brain endothelium), and hence is able to maintain the necessary supply of metabolic substrates and essential amino acids to the brain. However, whereas the mammalian brain endothelium shows negligible uptake of neurotransmitter molecules such as glutamate and GABA, there is a detectable uptake by the elasmobranch barrier (Abbott et al., 1988b). Glia in close association with neurons in most animal groups show transporters for amino acid transmitters including glutamate and GABA, so it is clear that the elasmobranch glia retain some 'gliallike' properties in spite of their barrier role. Since one of the functions of any blood-brain barrier is to exclude from the brain neuroactive substances circulating in the blood, it is likely that glutamate and GABA taken up by the

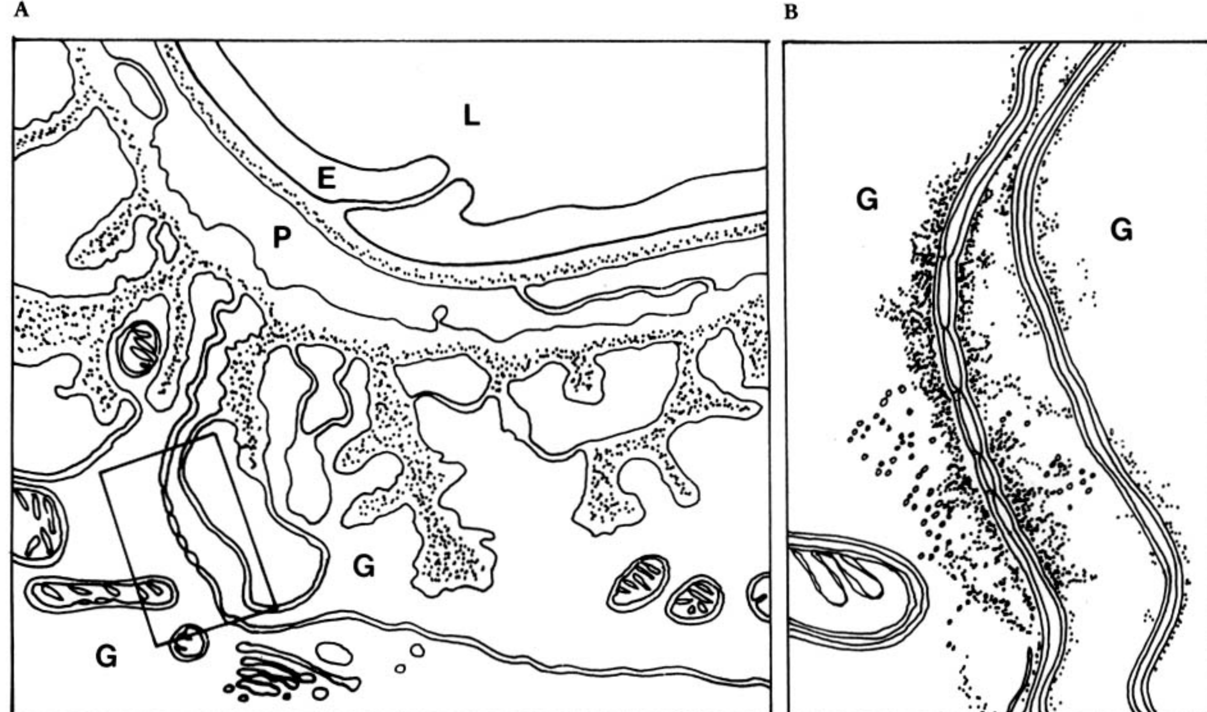


FIG. 6-11. (A, B) Perivascular glia (*G*) responsible for the glial blood-brain barrier in the skate *Raja* (Elasmobranchii), showing a tight junction with multiple membrane contact points (area outlined in Figure A, shown at higher magnification in Figure B). *L*, lumen; *E*, endothelium; *P*, pericyte; basal lamina material shown stippled in Figure A). Note the cytoplasmic densities and cytoskeletal elements associated with the junctional zone in Figure B. [Based on electron micrographs in Bundgaard and Cserr (1981), with permission.]

elasmobranch glial barrier will be broken down intracellularly, effectively preventing their penetration into the brain. Thus endothelial and glial barriers achieve the same end, but by different means (Abbott, 1991). Retinal glia in elasmobranchs show a similar distribution of potassium channels to those of teleosts and amphibia, suggesting they are also able to mediate potassium spatial buffering (Newman, 1988).

Amphibians and Reptiles

Glial Cells of Amphibians and Reptiles Have a Strong Radial Morphology. Amphibia and reptiles are poikilothermic (coldblooded) vertebrates, with a relatively simple nervous system, on a similar plan to that of higher groups such as mammals and birds, but with a less complex cortical structure and simpler organization of sensory and motor systems. Radial glia and tanycytes are retained in the adult (Monzon-Mayor et al., 1990). Within the retina are found the specialized "Müller" glial cells spanning from receptor layer to vitreous (see Chapter 4). Cells of the glioependyma form a system of channels in the spinal cord along which axons grow, in development and regeneration (Singer et al., 1979; see Chapters 27 and 60, this volume). In the optic nerve of the mudpuppy *Necturus,* glial cells form a surface layer, extending inward as partitions between the axon bundles (Figure 6-12). The relatively large size of amphibian glia, their accessibility, and their robustness has meant that amphibian glia have provided some excellent experimental preparations for electrophysiological study (see Chapters 31 and 37).

Birds

Glia of Birds and Mammals are Similar. The large and accessible eggs of birds have been useful in embryological and experimental studies. The complexity of the avian nervous system approaches that of mammals, and the similarities of anatomy make for ready extrapolation to the mammalian system (Roeling and Feirabend, 1988). However, many antibodies developed as markers for mammalian glia do not cross-react with birds, so the homologies are not always clear.

The Avian Embryo Gives Information About the Role of Glia in Development. The developing nervous system of the chick can be observed through the transparent membranes of the chorioallantois, and detailed

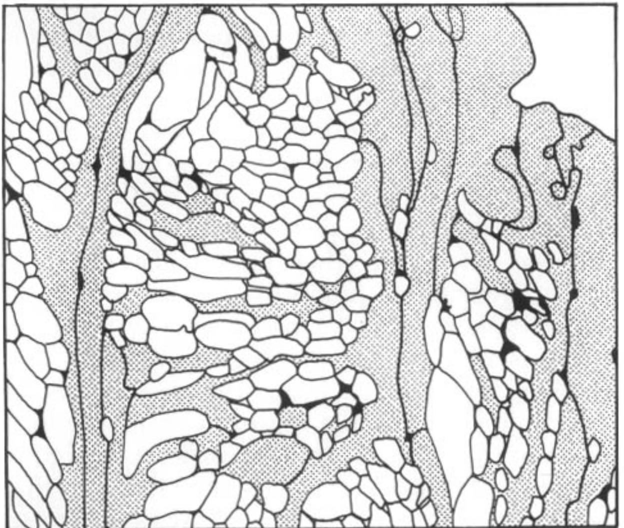


FIG. 6-12. Tracing of an electron micrograph of the glial cells (*shaded*) and axons at the surface of the optic nerve in the mudpuppy *Necturus* (Amphibia). The large size of the glial cells and their accessibility permits microelectrode penetration and physiological study. After enucleation, the axons degenerate, leaving a "glial nerve." [From Kuffler et al. (1966), with permission.]

observations and fine experimental manipulation are possible. Retrovirus-mediated gene transfer has been used to map the lineage of cells of the nervous system and show the common origin of astrocytes and neurons and their migration paths (Galileo et al., 1990; G. Gray and Sanes, 1991, 1992). The glia of the eye are particularly suitable for experimental manipulation.

Avian Glia Induce a Blood-Brain Barrier in Endothelial Cells. The avian system has also been instrumental in studies on blood-brain barrier induction. In higher vertebrates, including birds and mammals, the blood-brain barrier is formed by endothelial cells, but inductive factors from the nervous system are required. Grafting studies taking advantage of quail-chick chimeras showed clearly that if small pieces of donor brain were grafted into non-brain tissue of the host, the graft became vascularized by the host (Stewart and Wiley, 1981). The fact that the vessels in the graft developed blood-brain barrier properties is evidence for an inductive influence from neural tissue. Astrocytic clusters grafted onto the chorioallantoic membrane of the chick also became vascularized from the host, and again the vessels became tighter, evidence that astrocytes are responsible for at least part of the inductive influence (Janzer and Raff, 1987; see Chapter 50, this volume).

SUMMARY: COMPARISON OF MAMMALIAN AND NONMAMMALIAN GLIA

Glial Subtypes and Classification

In the earliest histological studies using classical silver and gold staining techniques, a great range of glial morphologies was described, but it was not known whether these represented different classes of glia. A general division into fibrous and protoplasmic astrocytes, oligodendrocytes, and microglia could be made on the basis of gross morphohology, location, and relation to neurons and blood vessels. The introduction of immunocytochemical methods and specific antibodies, together with molecular approaches to the question of cell lineage, allowed some generalizations about the relations and developmental sequence of glial types. The recent demonstration of differences in receptor and transporter expression on glial cells from different parts of the nervous system, has caused renewed interest in the possibility that individual glial cells may have phenotypes as varied as those of neurons, and in many cases this will be reflected in their morphologies. Lower vertebrate and invertebrate preparations offer the possibility of working with glial cells associated with identified neurons, something not yet practical for vertebrate glia *in situ*.

Role of Glia in Development and Regeneration

This survey of glia in different animal groups from invertebrates to birds has shown that glial cells play important roles in development by providing morphological landmarks and channels, by expressing surface molecules favourable for adhesion, and by secreting growth factors that stimulate proliferation and outgrowth. In lower animals many of these features are retained in the adult, permitting some regeneration, an ability largely lost in mammals. The strong macrophage involvement in the response to injury in lower vertebrates may also contribute to conditions favoring regeneration. Understanding the mechanisms used by lower vertebrates could lead to remedial strategies for neural repair in mammals, including humans.

Role of Glial Morphology in Regulation of the Neuronal Microenvironment

In both higher and lower groups, the morphology of glial cells confers certain properties that help them regulate the ionic environment. Thus the endfeet of perivascular glial cells, by "siphoning" potassium from neurons to the blood vessel wall may permit coupling between neuronal activity and cerebral

blood flow (Paulson and Newman, 1987). The vitread process of the retinal Müller cell, with its high density of inward rectifier potassium channels, permits operation of the potassium "spatial buffer" in the retina, and the fine astrocytic process apposed to the nodes of Ranvier may have an equivalent function in central nervous system white matter. The segregation of groups of axons and synapses by glial partitions may allow functional compartmentation and reduce "cross talk."

Role of Glial Cells in Synaptic Zones in Neurotransmitter Uptake and Recycling

In synaptic zones within the nervous system, space is at a premium, and nerve terminal processes are packed tightly together to make maximum use of the areas for membrane:membrane contact. The close association of glial cells with synaptic zones underlies two functions, morphological segregation of synaptic inputs (as in "glomeruli") and chemical segregation, by the presence of transport mechanisms capable of rapidly removing transmitter from the extracellular space. Glial cells may occupy as much as 50% of the vertebrate central nervous system volume and outnumber neurons by 10:1 (Nicholls et al., 1992); this implies a much greater surface area of glial than neuronal membrane, an optimal arrangement for providing transporters on glial membranes close to synaptic sites.

Role of Glial Cells in the Blood-Brain Barrier

All higher animals possess a blood-brain barrier, which is formed by glial cells in invertebrates and elasmobranchs, and by endothelial cells in higher vertebrates. Evolutionary and developmental considerations suggest that a glial barrier is the primitive condition, and the shift to an endothelial barrier is one aspect of the greater division of labor between cell types seen in the central nervous system of higher animals. Where the barrier is now endothelial, glial cells are still important in inducing and maintaining the barrier, which makes sense of the close association between glial endfeet and vessel, and helps explain breakdown of the barrier in some glial tumors and other central nervous system pathologies where the inductive influence is disturbed (reviewed in Abbott et al., 1992b).

Polarity and Barrier-Forming Potential of Mammalian Glia

Where glial cells form an epitheliallike layer in invertebrates, for example, in the perineurium of arthropod ganglia and connectives, and the perivascular barrier glia of the cephalopod brain, there is a clear polarity to the cell, the basal surface secreting a prominent basal lamina, and the apical end frequently demonstrating surface specializations, such as elongated processes running into the nervous tissue. Where cell:cell junctions occur, they are generally close to the apical end of the lateral intercellular space, as in other epithelia. The glial cells and undifferentiated primordial cells of the vertebrate central nervous system express clear polarized properties in certain locations, for example, the ependyma, glia limitans below the pia, retinal pigment epithelium, choroid plexus epithelium, radial glia and tanycytes, and Müller cells of the retina (reviewed in Abbott et al., 1986a). Astrocytes that have lost contact with the pial or ventricular surface of the vertebrate central nervous system may nevertheless retain a functional polarity (e.g., basal endfeet on blood vessel and apical processes associated with neurons) (Bradbury, 1979). As better morphological markers are developed for looking at regional differences in glial membranes, this functional polarity is likely to take on increasing significance, showing that differences in function are not only seen between different glial types, but also between different cells within a class, and between different parts of the cell membrane of a single glial cell (Reichenbach, 1989; Holder et al., 1990). Throughout the history of glial studies, the close relations between glial morphology and glial function have been a continuing and productive theme.

Relative Roles of Glia and Hematogenous Cells

In teleost fish, the brain contains large numbers of macrophages that service myelin and leukocytes associated with ependymal tanycytes and radial glia; the brain has good regenerative capacity. In higher vertebrates, hematogenous cells are largely excluded from the brain, myelin debris is removed by the resident macrophages (microglia) and by astrocytes, and there is minimal capacity for regeneration. The transfer of function from hematogenous cells to macroglia and microglia is associated with increasing sophistication of the immune system, and increasing isolation of the nervous system from the blood compartment. This evolutionary pattern may have been necessary to reduce the risk of autoimmune attack on the nervous system, but the ability to repair central nervous system damage may have been sacrificed in the process (Dowding and Scholes, 1993).

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II Lineage

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7 Methods of studying glial cell lineage

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Glial cells, like neurons, are derived from embryonic neuroepithelial cells. In the central nervous system, these neuroepithelial cells belong to the neural plate; in the peripheral nervous system they are the cells of the neural crest and the placodes. The neuroepithelium is initially a simple pseudostratified epithelium in which all the cells are dividing and all apparently behave the same. As development proceeds, however, the behavior of the neuroepithelial cells changes. Some cells stop dividing and begin to migrate from the neuroepithelium. These are the first neurons, and in most areas of the neuroepithelium their production dominates the embryonic period. The onset of gliogenesis is more difficult to identify precisely, but for most areas of the nervous system the main period of glial production follows neurogenesis, the production of oligodendrocytes usually following that of astrocytes. The cerebral cortex of a newborn rat, for example, is composed predominantly of neurons, but 2 weeks later there are many more glia than neurons (Parnavelas et al., 1983). It is also generally true that the glial cells are not generated directly from the neuroepithelial cells, but rather via intermediate precursor cells, such as those that make up the subventricular zone in the cerebral cortex (Wood and Bunge, 1984).

This pattern of development poses a number of problems. The neuroepithelial cells have the potential to generate glia, yet this potential is not expressed until most of the neurons have been produced. Our ultimate goal, of course, is to understand how these processes are controlled in cellular and molecular terms, but the aim of the studies discussed in this chapter is more modest. A first step toward understanding nervous system histogenesis is to understand what "fate" decisions are being taken by cells at any given point in development, and how these decisions alter their behavior. This brings us to the topic of cell lineage.

Cell lineage is concerned with how different cell types are related. Its significance becomes clear if we consider the generation of cellular diversity in terms of two alternative scenarios. The neuroepithelial cell population could be made up of cells that are all pluripotential; that is, each cell is capable of generating each of the different cell types. If so, then each neuroepithelial cell could generate neurons for a period, then switch to the production of astrocytes (or their immediate precursors), then switch to the production of oligodendrocytes. If this were the case, the mechanistic problem would be: how is the switching controlled? The second possibility, however, is that neuroepithelial cells themselves are of different types, each dedicated to the production of one type of cortical cell. Thus, initially a neuronal precursor population would be actively generating neurons, while the glial precursors would not produce differentiated cells at all. They would presumably only divide to give more cells like themselves. Later, the neuronal precursor cells would become silent or disappear, and the astrocyte precursors would become activated, followed in their turn by the oligodendrocyte precursors. In this model, there is no switching of cell fate at all, and it would be pointless asking at late stages of development what controls whether a cell gives rise to a neuron or a glial cell, since no precursor cell was making that choice at that time. In order to understand which of these two models (or any of the other theoretically plausible alternatives) is true, we need to know the lineage relationships of the neural cell types. When, for example, do precursor cells cease to generate both neurons and glial cells? Is it late in development as the first model would predict, or early as predicted by model two? To rephrase the question in a testable form: how many different types of precursor cell are there; what do they generate; and how do they change with time?

In this chapter, I describe how cell lineage and the related fields of study are being approached, both conceptually and experimentally, and the picture that is emerging from these studies.

CELL LINEAGE LABELS

The simplest way to discover what a precursor cell will generate is to label it, allow it to develop un-

disturbed, then look at what the marked cell has produced. Two methods have been developed to do this in vertebrate embryos: the direct injection of lineage labels and retroviral vectors.

Injection of Lineage Labels

The most direct method of labeling a cell is to inject it with a marker, usually either a fluorescent compound or an enzyme that can be detected histochemically. A recent innovation has been to use a caged fluorescent marker that requires laser activation, but this has so far only been applied to the study of invertebrates (Vincent and O'Farrell, 1992). As the precursor cell divides, the cells it generates will inherit some of the labeled cytoplasm and so also be labeled. This technique was originally devised to label precursor cells in invertebrates (Weisblat et al., 1978), where the cells are large and easily accessible. It was subsequently applied to amphibian embryos, which also have the advantages of size and accessibility (Jacobson and Hirose, 1978). One of the notable recent technical achievements in this field has been the adaptation of this technique to label the very small precursor cells of amniotes (Bronner-Fraser and Fraser, 1988; Fraser et al., 1990; Lawson et al., 1991). This has contributed to our knowledge of cell lineage in the retina (Holt et al., 1988; Wetts and Fraser, 1988), which will be discussed below, and provided the single most dramatic finding in central nervous system lineage, the lineage compartments of the hindbrain (Fraser et al., 1990).

The principal advantage of the direct labeling approach is its simplicity and its certainty; the number and position of the labeled cells are known precisely. There are, however, a number of potential disadvantages. For instance, the injected substance might be toxic, or the cell might be damaged by the injection. In addition there are two major limitations. First, only precursor cells that are accessible to direct injection can be studied. This currently excludes all neural plate precursor cells in mammalian embryos. The chick neural plate has proved more tractable, however, because of its larger size and accessibility (Lunn et al., 1987; Bronner-Fraser and Fraser, 1988; Fraser et al., 1990). Second, as the clone of cells derived from the injected cell grows, the label becomes more and more dilute. Eventually, the point is reached when it becomes undetectable. Since this point is likely to be before development is complete, this can severely restrict the lineage relationships that can be studied. This presumably explains in part why most the studies using this technique have not addressed the issue of glial lineage in any depth.

Retroviral Vectors

A second way of marking precursor cells is genetically. The idea is that, rather than insert a marker substance by injection, a precursor cell is marked by the introduction of a gene. This has been achieved in insects using x-radiation-induced mitotic crossover (Ready et al., 1976; Lawrence and Green, 1979). Similar methods have been used in mammals (Wikler and Rakic, 1991), but not to study glial lineage as far as I am aware. More recently, genetic labeling has been achieved in mammalian embryos with retroviral vectors.

Retroviruses are a naturally evolved means of gene transfer. Consequently, they are accurate, efficient, and they operate under normal physiological conditions. A normal wild-type retrovirus gets into a cell by receptor-mediated endocytosis (Figure 7-1). Once inside, the RNA genome of the virus is reverse-transcribed to give, ultimately, a double-stranded DNA provirus. This provirus gains access to the infected cell's chromosomal DNA, into which it integrates. For reasons that are improperly understood, integration can only take place into cells that are moving through the cell cycle; postmitotic cells cannot be labeled.

There are two important consequences of viral integration. First, genes encoded by the virus will be transcribed and translated using the cell's normal machinery. A retrovirus carries its own powerful promoter elements (although these can be replaced with others). Thus, it comprises an independent transcriptional unit, which has evolved to give high levels of expression in a wide range of cell types. Second, when the cell carrying the integrated provirus divides, the viral DNA will be inherited unchanged by both daughter cells; it has become a permanent, genetic marker of all the progeny of the labeled cell.

There is, however, a complication associated with integration. Recent work has shown that when a precursor cell is infected, the retroviral marker is inherited by only one daughter at the next division (Hajihosseini et al., 1994). This is apparently because the retroviral DNA integrates into the chromosomal DNA of the infected cell as that cell divides at M phase (Roe et al., 1993). Since the chromosomal DNA has already replicated at this point in the cell cycle, only one daughter cell can inherit the provirus (Figure 7-2A). This has important consequences for retroviral lineage labeling. At a minimum, it means that the lineage has been labeled one cell cycle later than might have been anticipated, but if the precursor cell is dividing asymmetrically, like a stem cell, the consequences are more profound be-

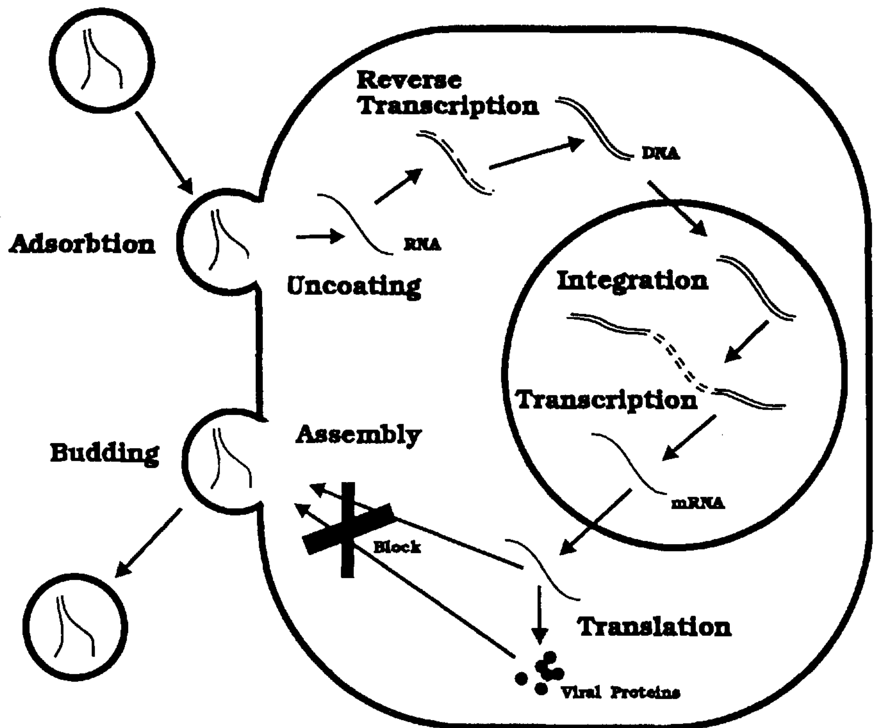

FIG. 7-1. The life cycle of a wild-type retrovirus. A retrovirus is an encapsulated structure containing two copies of a single-stranded RNA genome. It adsorbs onto the surface of a cell by means of an interaction between its envelope glycoprotein and its cellular receptor, and so enters the cell by means of receptor-mediated endocytosis. Inside the cell, the vial RNA is reverse transcribed by a series of steps into linear double-stranded DNA, and this form of the virus integrates into the host cell chromosomal DNA at an apparently random site. Once integrated, the viral genome is transcribed and translated using the cell's normal machinery. In a wild-type virus, the life cycle is completed by the combination of retroviral genomic RNA and proteins to assemble new viral particles that bud from the surface of the cell. In retroviral vectors of the type used for cell lineage marking, however, the life cycle is blocked at this point and new particles cannot be formed.

cause the two daughters of a stem cell can have quite different fates (Figure 7-2B, C). One daughter will become a stem cell similar to the mother cell. If this cell inherits the provirus, then the lineage will be simply be labeled one cell cycle later than was anticipated, just as for a symmetrically dividing lineage (Figure 7-2B). The other daughter cell, however, is likely to be a committed cell of some type, either an intermediate glial precursor cell, or (in the ultimate case) a postmitotic neuron. If this cell inherits the virus, the labeled clone of cells will be restricted in their fate, perhaps limited to a single neuron (Figure 7-2C). This restricted outcome will be the result of approximately 50% of infections of asymmetrically dividing stem cells. This presumably explains why so many one-cell neuronal clones have been observed in retroviral lineage experiments (Price and Thurlow, 1988; Walsh and Cepko, 1988; Moore and Price, 1992). This twist is quite fortuitous in that it helps to distinguish symmetric from asymmetric modes of division, but it complicates the interpretation of lineage data in that undetermined precursor cells can generate clones that appear restricted.

Returning to the life cycle of a wild-type retrovirus (Figure 7-1), the final step is the assembly of the viral proteins together with viral RNA to generate new viral particles, which bud from the cell surface. The retroviral vectors that are used as lineage labels, however, do not have this facility. They can infect cells, integrate, and express, but they have been engineered so that they cannot produce the next generation of viral particles. The life cycle is blocked at the stage indicated in Figure 7-1. This is of obvious importance if vectors are to be used as lineage labels. It would be useless if a virus used to mark a cell and its progeny were to spread the genetic label to neighboring cells. There would be no way to distinguish the progeny of the original cell from those secondarily infected. The lineage vectors have the added advantage that they encode genes that can be recognized histochemically. The *lacZ* gene encoding the enzyme β-galactosidase has been a favorite (Sanes et al., 1986; Price et al., 1987), although vectors encoding the alkaline phosphatase gene look set to become popular in the future (Fields-Berry et al., 1992).

The viral vectors that have been used for lineage studies fall into three categories: ecotropic mouse viruses, amphotropic mouse viruses, and chick viruses (see Stoker (1993) for a comprehensive list). The principle difference between them is their host range. Ecotropic mouse viruses, mostly based on Moloney murine leukemia virus (MLV), infect cells of mouse and rat but few other species. Amphotropic viruses carry the same range of genomes, but have a different coat protein, which allows them to infect a broader range of species, including chicks, rabbits, primates (including human) as well as rodents. The chick viruses that have been used for cell lineage studies are derived from the Rous sarcoma virus (RSV).

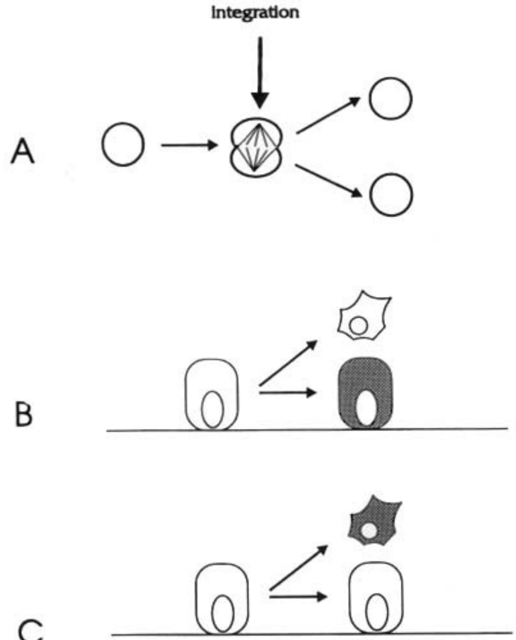

FIG. 7-2. Retroviral integration. (A) Although, as far as we are aware, a virus can infect a cell during any phase of the cell cycle, the process of integration only takes place as the cell divides. Thus the genetic labeling only occurs at this point. It follows, therefore, that when a cell is infected, only one of its daughter cells will actually inherit the genetic label. If the lineage is a symmetric one such as that shown here, in which both daughters are alike, then the effect of this delay in labeling is quantitative rather than qualitative. Effectively, the lineage is labeled one cell cycle later than might have been thought to be the case. (B, C) The consequence for an asymmetrically dividing precursor cell. This cell divides to give one daughter that is a stem cell the parent cell, and a second daughter that takes on a specific fate. In the context of the nervous system, this latter cell could be a post-mitotic neuron or a glial cell (or glial cell precursors), which will continue to divide, but only to give other glial cells. If, by chance, the stem cell daughter inherits the label (Figure B), then the lineage is labeled, just as with the symmetrically dividing precursor cell in Figure A. If the other daughter inherits the viral DNA, however, (Figure C) then the labeled clone is composed only of the differentiated progeny, which could be a single cell. Thus roughly 50% of stem cell infections will label restricted progeny, even though the originally infected stem cell might have a broad developmental potential.

Retroviruses avoid a number of the problems associated with injected lineage labels. They need not be injected directly into cells so the size of the cell becomes irrelevant and there is no danger of injection damage. They are known not to have any toxic effects on the infected cells, although this cannot be assumed for any novel gene they might be engineered to carry. They need only be introduced into the vicinity of dividing cells for the cells to become infected, so problems of inaccessibility of precursor cells are reduced. Most significantly, the genetic label never gets diluted however large the clone derived from the infected cell becomes.

Unfortunately, there are also disadvantages. One problem is that, although the marker gene will be inherited genetically, it will only be detected if it is expressed. If for any reason a cell were to turn off expression, it would disappear from the analysis. The extent to which this theoretical problem is of practical significance is still unclear; the endogenous enhancer of MLV is inactive in all cells of the preimplantation mouse embryo, and in undifferentiated embryonic stem cell lines (Jaenisch et al., 1975). From about 10 days of gestation, however, cells of all germ layers express genes driven by this enhancer (Savatier et al., 1990), and, although some cell lines do not express in a predictable way, the evidence for normal cells suggests reliable long-term expression for the most part.

The main disadvantage of the retroviral method, however, is that it has neither of the two principal certainties of the injection method: the experimenter knows neither precisely how many cells were infected, nor exactly where they were. Both of these have to be inferred in the final analysis from the distribution of labeled cells. This can lead to two types of error, which Walsh and Cepko (1992) call "lumping" or "splitting." If two closely neighboring cells became infected, two labeled clones would inhabit overlapping territory and so be "lumped" together as one. Alternatively, a labeled clone could generate a migratory cell that left the site of infection and generated a second cluster of labeled cells some distance from the main body of labeled cells. Because these two halves of the clone had become split, they might be mistakenly interpreted as being two distinct clones. As we shall see later, these are real problems in the interpretation of retroviral lineage data, for which there are as yet no easy solutions. What is required is an unequivocal means of distinguishing clones. So far, the only proposed solution to this problem has come from Walsh and Cepko (1992). Their method uses 100 different viral variants that can be distinguished by polymerase chain reaction, and this they have applied to the study of cell lineage in the cerebral cortex (see below). Whether this will provide the ultimate solution to the errors of interpretation, however, is not clear. The polymerase chain reaction method has its own problems of interpretation, as has been discussed elsewhere (Kirkwood et al., 1992), but equally significantly, this method is far from simple. Generating so many different viruses is a considerable amount of labor, especially as each virus has to be of precisely equal titer. This may limit the extensive adoption of the technique. Currently, several laboratories are study-

ing glial cell lineage, successfully working within the limitations of the original technique, but the Walsh and Cepko method, or something like it, is certainly required if retroviral lineage labeling is to fulfil its full potential.

These problems notwithstanding, with the retroviral technique we have the potential to label almost any precursor cell in the embryo by injecting virus into its vicinity (see Price, 1993a for a practical guide). Once labeled, the cell can be left for as little time as 2 days (the minimum required for expression of the virus) or as long as the lifetime of the animal before the infected tissue is analyzed to discover what has become of the labeled cell. This approach has now been applied to the retina (Sun and Wekerle, 1986; Turner and Cepko, 1987), cerebral cortex (Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988, 1992; Austin and Cepko, 1990; Grove et al., 1993), hippocampus (Grove et al., 1992a) and striatum (Halliday and Cepko, 1992) of rodents, and the optic tectum (Gray et al., 1988; Galileo et al., 1990; Gray and Sanes, 1991; Johnston and Van der Kooy, 1992), spinal cord (Leber et al., 1990), and neural crest (Frank and Sanes, 1991) of birds.

OTHER METHODS OF FOLLOWING CELL FATE

These two methods—the injection of lineage tracers, and retroviral vectors—have been the two most prominent methods for studying cell lineage in vertebrates over the past decade. The study of fate determination in general, however, has involved a number of other types of study. Before we consider those, we need to consider how different approaches relate to each other conceptually.

The study of cell lineage is the study of presumptive fate. By labeling a precursor cell *in vivo*, we discover what it will generate if left undisturbed. A different experiment would be to label the cell, then move it to a different environment—another part of the embryo for example, or into a tissue culture dish. In the new environment, it might behave differently. We can imagine circumstances under which it might show either a greater or a lesser potential than it would have shown undisturbed. For example, a precursor cell might have the potential to generate, say, both astrocytes and oligodendrocytes, but only one cell type might arise *in vivo* because to generate the other, the cell must be stimulated by a particular factor. The factor might be missing from the cell's natural environment, but present in the alternative environment. Conversely, the factor might be present in the natural environment so that the precursor cell generates both oligodendrocytes and astrocytes, but absent from the new environment. Therefore cell lineage—the study of the undisturbed cell—does not necessarily tell us the cell's full potential, whereas following a cell in a foreign environment, tells us something of its developmental potential without telling us the cell's presumptive fate.

In parallel with the distinction between presumptive fate and developmental potential, we also need to observe that between studies of single cells and cell populations. Just as a single cell can be labeled and its fate followed, so a population of cells can be labeled and their collective fate recorded. A study of the presumptive fate of cells is termed a *fate map,* a description of the contribution made by populations of cells in the embryo to the final adult structure. This is analogous to the study of cell lineage, but fails to address the questions we set ourselves earlier because it does not tell us what individual cells are doing. We know that if we labeled neuroepithelial cells as a population, they would give rise to neurons and glia. The question is: Are individual cells generating all the cell types, or just some of them?

STUDIES OF CELL POPULATIONS

As with lineage studies, the principal problem in studying cell populations is how to label them. For many years embryologists were limited to those cells that fortuitously carried natural markers, such as yolk granules or pigments, or that could easily be labeled with vital dyes such as neutral red. Currently, however, there are a wide variety of labeling techniques available. Several can be applied to normal *in vivo* development, and many others can be used to distinguish cells that have been transplanted into an alternative environment.

Labeling Populations In Vivo

The problem with techniques for labeling normal cells *in vivo* is that they generally give the experimenter little control over the precise population to be labeled. Three examples that are otherwise very different are fluorescent labels, rodent chimeras, and nucleotide labels, such as thymidine (TdR) and bromodeoxyuridine (BrdU).

Fluorescent Labels. Labeling individual cells by the injection of dye substances has already been described. Populations can also be labeled without the necessity for individual cells to be injected. Fluorescent labels such as CFSE (Bronner-Fraser, 1985) or the carbocyanine dyes (Honig and Hume, 1986;

Godeman et al., 1987) label cells by passive uptake or incorporation into the cell membrane, respectively. The carbocyanine dyes have revolutionized the tracing of axonal pathways, but have also been useful in labeling radial glial cells. In particular, this has led to the best evidence to date that radial glia give rise to protoplasmic astrocytes (Voight, 1989).

Rodent Chimeras. There are a variety of ways of generating chimeras—individuals whose cells are a mixture of two different genotypes. The most common in studies of brain development is to aggregate morulae from two different mouse strains to give an apparently normal individual, made up of cells from both strains (Herrup et al., 1984; Herrup and Sunter, 1986; Goldowitz, 1987, 1989; R. Williams and Goldowitz, 1992b). The proportion from each strain varies between individuals and depends primarily on the proportion of cells from each morula that happened to contribute to the embryo rather than to the extraembryonic tissues. The two strains are selected because their cells can be distinguished histochemically. Thus, by staining sections of brain, the contribution made by each strain to any particular brain structure can be determined.

The problem with this approach has always been one of interpretation. Since the strains were combined so early in development, both contribute to all parts of the embryo, but there are local distortions in both the proportions and distributions of the cells derived from each strain. These distortions have been translated into counts of the number of founder cells of different areas (Herrup et al., 1984; Herrup and Sunter, 1986), and into evidence for developmental compartments (Crandall and Herrup, 1990; R. Williams and Goldowitz, 1992b). These interpretations have been criticised on methodological grounds (Mead et al., 1987; Jennings, 1988). They also tend to ignore artifacts such as the apparent tendency of cells of different strains to differentially adhere (Goldowitz, 1989).

These limitations notwithstanding, chimeras have proved a useful way of following the fate of glial cells, particularly in the study of microglia. By taking bone marrow from one rodent strain and transferring into an irradiated host of another strain, for example, experimenters have been able to address the question of whether microglia cell populations were derived from host or grafted tissue (Lassmann et al. 1993). This relates to the issue of origin of microglial cells, discussed in Chapter 10.

Nucleotide Labeling. The labeling of cells with TdR has a much more established pedigree. If TdR (or, more recently, BrdU) is introduced into an embryo, it is taken up by all the dividing cells, which incorporate it into their nascent DNA and pass it on to their progeny. Cells that continue to divide dilute out the labeled TdR at each subsequent S phase, but cells that become labeled immediately before their final division carry the radiolabel into adulthood. This has been an immensely important technique for establishing the timing of production of different cell types, particularly neurons, but also glial cells such as those of the optic nerve (Skoff et al., 1976a, 1976b; Skoff, 1990). It has also provided a means of labeling cells for transplantation so that they can be distinguished from host cells (Lindsay and Raisman, 1984).

Nucleotide labels are not good lineage labels because they label all dividing precursor cells indiscriminately, and therefore provide no means of distinguishing the progeny of different cells. This does not mean that studies of populations of cells labeled in this way cannot be extremely informative. A good example is the study of Watanabe and Raff (1988), who showed that retinal astrocytes are derived from the optic nerve rather than being indigenous retinal cells. In part, this conclusion was derived from experiments in which retinal populations from different developmental stages were cultured together. By first labeling one population with BrdU, Watanabe and Raff were able to distinguish between the progeny of the different populations. Like the study of Espinosa de los Monteros et al. (1993) discussed below, this is good example of how single-cell labeling need not be necessary in studying fate if all the members of a population behave similarly with regard to the particular feature being studied. As we shall see later in our discussion of retinal lineage, the Watanabe and Raff result complemented the retroviral lineage data nicely to give a more complete picture of retinal lineage.

Labeling Transplanted Cells

In considering cell transplantation, we are straying rather far from our subject of cell lineage. Not only are we considering the development of cell populations rather than single cells, we are also considering cells whose development has been perturbed by transplantation. Nonetheless, much of what we know of cell fate comes from such studies, and in many cases they have been immensely informative. Perhaps the single most significant in this regard is the chick-quail chimera system.

The Chick-Quail System. First described by Le Douarin, chick-quail chimeras have proved to be probably the most successful approach to cell label-

ing in modern embryology (see Le Douarin, 1982). The basis of the technique is that chick and quail cells can be distinguished by the appearance of their nuclei after Feulgen staining. This means that cells transplanted from one species into the other can be distinguished from the indigenous cells. Thus a piece of neuroepithelium, for example, can be transplanted from a quail embryo into the equivalent site of a chick at the same embryonic stage. After the animal has developed, the contribution of the transplant to the developed structure can be determined. The result of such an isochronic, isotopic transplant is not strictly a fate map because cells have not been labeled undisturbed, but transplanted between embryos. But because exactly equivalent cells have been transplanted, they are assumed to develop exactly as the undisturbed cells would have done, and the result is considered to be a fate map.

Heterotopic and heterochronic grafts are also possible; that is, cells can be transplanted to inappropriate positions in the embryo, and between embryos at different developmental stages. Thus, this versatile approach can give information about the developmental potential of cells, as well as their presumptive fate. As a consequence, we know a great deal about the fate of the avian neural crest (including peripheral glial cells) particularly from the work of Le Douarin. The approach is being turned increasingly to the central nervous system to generate fate maps of the avian neural plate, and to address questions regarding the positional specification of the neural plate.

This technique has told us a considerable amount about the derivation of peripheral nervous system glia. All peripheral nervous system glia are derived from the neural crest (Le Douarin et al., 1991). This includes satellite cells, both myelinating and nonmyelinating Schwann cells, and the cells of some cutaneous receptors (Halata et al., 1990). The only exception is the sheath cells of the ventral root, which have been observed (also using chick-quail chimeras) to arise directly from the ventral neural tube (Lunn et al., 1987). Some workers, however, doubt this observation (Le Douarin et al., 1991). Little has been done specifically on central nervous system glia using this technique, although some observations have been made in passing. For example, Hallonet et al. (1990) have suggested that the glial cells of the avian cerebellum are derived directly from the neuroepithelium, not from the external granular layer.

Other Labeling Techniques for Transplanted Cells. There have been too many different approaches to labeling cells for transplantation for them all to be considered in detail here, especially since most are not specifically concerned with following cell fate. Some of the methods that have already been mentioned have also been used to label cells for transplantation, for example, retroviruses (Emson et al., 1990) and thymidine (McConnell, 1985, 1988; Emmett et al., 1991). Astrocytes have been labeled with fluorescent beads to aid their recognition following transplantation (Emmett et al., 1991). O-2A cells have been labeled with the fluorescent dye, fast blue (Espinosa de los Monteros et al., 1993).

A technique that has been prominent in glial cell biology has been to transplant pieces of tissue either from mutant animals into wild type, or vice versa (Duncan et al., 1988; Rosenbluth et al., 1990; Gansmuller et al., 1991). A number of mutants, such as the *shiverer* mouse and the myelin-deficient rat, are deficient in oligodendrocytes. Thus either explants of normal tissue (or isolated oligodendrocytes or Schwann cells from normal animals) are easily recognized following transplantation into mutant hosts, since they are the only source of myelinating cells. Conceptually similar is the approach of Blakemore and his colleagues in which a region of spinal cord is experimentally demyelinated then used as a target tissue for the implantation of glial cells (Blakemore and Franklin, 1991).

Most of these studies of oligodendrocytes have primarily addressed the process of myelination, rather that the process of cell fate allocation *per se*. An exception is the study of Espinosa de los Monteros and colleagues (1993) where cultured O-2A cells were labeled with the dye, fast blue, and transplanted into neonatal brain to see whether they generated astrocytes or oligodendrocytes. O-2A cells generate both cell types in tissue culture, but evidence that they produce astrocytes *in vivo* has been lacking (see Chapter 8 for a discussion of this issue). Since the transplanted cells generated oligodendrocytes but not astrocytes, this was taken as support for the contention that the O-2A cell is primarily an oligodendrocyte precursor cell *in vivo*.

Note that in this study, the transplanted cells are cultured cells, operationally defined as all belonging to the same type. Had the result been that the transplanted cells also generated astrocytes, the question would have arisen whether contaminating astroblasts could have contributed that cell type, or whether all the defined cells were really identical in developmental terms. In the event, that consideration was unnecessary, but we see how the conclusions from this study differ inherently from those that follow from single-cell, cell lineage studies. Similarly, the cells in question were derived from neonatal animals, cultured for approximately 2 weeks

in total. This is therefore a heterochronic graft, which limits the conclusions that can be drawn about the fate of undisturbed neonatal O-2A cells. These considerations highlight nicely the contrast between a study of single undisturbed cells and transplanted populations.

Finally, in this section we should consider the transplantation of cell lines. Primary cells taken from embryonic brain usually have a finite lifetime *in vitro*. An oncogene, however, can immortalize precursor cells, that is, induce them to divide *in vitro* for considerably extended periods. This allows time for individual precursor cells to expand into clones of many millions of cells, which can be labeled with retrovirus and transplanted into a normally developing central nervous system (Renfranz et al., 1991; Snyder et al., 1992). Since the transplanted cells were derived from a single transformed precursor cell, all the progeny of the transplanted cells are clonally related. As such, they represent the developmental potential of the founder cell. The question that is begged, of course, is how this potential is related to that the cell possessed before it was transformed.

A second question is how typically this potential of the transformed cell reflects the true potential of a cell *in vivo*, and if it does, then how representative this is of the starting population of precursor cells. Only a tiny proportion of cells become immortalized by the oncogene, and there is no reason to believe that all precursor cells have an equal tendency to become immortalized. So, in addition to the proviso of all transplantation studies regarding the relationship between presumptive fate and developmental potential, with cell lines there is the doubt that the developmental potential is a genuine one.

The true value of cell lines lies not in cell lineage, for which they are a poor substitute, but in the broader issue of fate determination. Consider the study of Renfranz et al. (1991). They immortalized a cell line derived from rat hippocampus and injected the cells into, among other sites, the cerebellum. There the cells gave rise to granule cells and Bergmann glia. This is a remarkable finding, which potentially has great significance for the therapeutic application of cell transplantation, especially since there seems no reason to believe that the cells generated were not genuine, functional granule cells and Bergmann glia. But what of cell lineage? The study of Hallonet et al. (1990) suggest that, in avian species, granule cells and Bergmann glia are derived from different areas of the neuroepithelium. Assuming the same to be true of rodents, we can conclude that no normal neural precursor cell ever expresses the potential demonstrated by this cell line. The juxtaposition of these studies of cell lineage and cell transplantation have, therefore, highlighted an important set of questions. Either neural precursor cells (or at least some of them) have a considerably broader potential than they will ever express—a potential that even crosses the major neuromeric divisions of the brain—or the process of immortalization has induced a primitive neurogenic fate, never found *in vivo*. If we could settle between these alternatives, we would be a good deal closer to understanding fate determination in the nervous system.

Means of Studying the Progress of Differentiation

There are other means of studying populations of cells that, though not studies of cell lineage *per se*, provide relevant information, and are indeed often confused with studies of lineage. The study of cell differentiation is a particularly important example. As cells differentiate, they synthesize specific macromolecules that can be considered markers of that differentiated state. Glial fibrillary acidic protein, for instance, is specifically made by astrocytes in the central nervous system, and is a marker of that cell type. A related set of intermediate filament proteins, neurofilaments, are likewise specific for neurons. Two obvious developmental questions arise: when in ontogeny is each marker first expressed; and what is the sequence in which markers of a cell type begin to be expressed?

These questions lead us to a description of the process of differentiation, and as such have proved very useful in the study of many cell types, including glia. Such studies do not, however, say anything directly about cell lineage, or the timing of commitment, although they may allow such conclusions to be inferred indirectly. For instance, many markers first appear in precursor cells. The temptation is to assume that these differentiation markers are also markers of lineage; to assume that is, that a marked population of precursor cells is restricted to the production of the cell type in question, and that this restriction of developmental potential occurred at the time of appearance of the marker. Neither of these conclusions can be defended logically. Restriction of gene expression in precursor cells, which presumably underlies the appearance of the marker, might be quite different from restriction in mature cells. So it proves with the markers I have mentioned. Both glial fibrillary acidic protein and neurofilaments are found in some neuroepithelial cells (Levitt et al., 1981; Tapscott et al., 1981; Bennett and DiLullo, 1985). Since this expression is only in some cells, this certainly tells us that there is hetero-

geneity among these precursor cells, and that is important in adjudicating between our various lineage models. Nonetheless, this observation has not told us what these cells will become, and the danger of believing the contrary becomes apparent when we consider that these markers are known to be expressed by cells that take on a different fate. Some embryonic heart cells, for example, express neurofilaments (Bennett and DiLullo, 1985); some young oligodendrocytes express glial fibrillary acidic protein (Choi and Kim, 1984; Ogawa et al., 1985). The important point is that tracing the appearance of markers in differentiating cells tells us something about the process of differentiation; but the appearance of markers in dividing cells does not reveal when lineages diverge nor when cell fate decisions are made.

Markers of differentiation are nonetheless very useful tools so long as these traps of interpretation are avoided. They can be particularly useful in defining populations of cells. A good example is the monoclonal antibody A2B5 and its role in defining the O-2A cell. A2B5 is not monospecific in as much as it recognizes glycosylation groups found on a number of proteins and lipids. Nonetheless, among neonatal rat optic nerve cells it recognizes a population of cells, which can become either oligodendrocytes or astrocytes in culture, depending on the conditions (Raff et al., 1983; see Chapter 8, this volume). Because almost all of this population could take on either fate, each cell must have been bipotential, regardless of the heterogeneity of the molecules recognized by the antibody, or of the subsequent expression pattern of these antigenic determinants. This conclusion was crucial in defining the potential of the O-2A cell in culture (Raff, 1989).

Studying Fate in Tissue Culture

Overwhelmingly, the most convenient means of studying developmental potential is in tissue culture. There is an almost infinite variation of types of cultures now available for the study of glial cells, and each have their relative advantages and disadvantages. All, however, have a number of advantages over studying cells *in vivo*. Cells in culture are more accessible, and more manipulable. They can be transferred from one environment to another; they can be injected; they can be sorted with a cell sorter; they can be photographed with a time-lapse camera. All of these are more difficult *in vivo*. Most importantly, the environment of a cell can be controlled in culture in a fashion that is impossible *in vivo*. Cells can be grown on different substrates, in the presence of different growth factors or drugs, alone or with other cells.

Different culture systems trade some of these advantages for verisimilitude. In slice and reaggregate cultures, cells retain some of their normal histotypic relationships, whereas in dissociated cultures, all such relationships are assumed to be lost. The ultimate is single-cell culture; the cell's environment is completely controlled but completely artificial.

Cell Labeling in Tissue Culture

Even in culture, the central problem of cell lineage studies remains: how can the fate of single cells be analyzed in the presence of many other cells? In general, the methods that have already been discussed in relation to *in vivo* experiments are also available for tissue culture studies, plus others that are only applicable to tissue culture. I know of no studies in which lineage labels have been injected into glial precursor cells in culture, but retroviruses are beginning to be applied to tissue culture studies. They have shown that in the embryonic cerebral cortex, separate precursor cells generate each different cell type, and that a further precursor type generates both neurons and oligodendrocytes (B. Williams et al., 1991). They have shown oligodendrocyte and astrocyte precursors to be separate in the neonatal cortex (Vaysse and Goldman, 1990), and multiple astrocyte precursor types in the neonatal spinal cord (Miller and Szigeti, 1991). In addition, many of the means of labeling cell populations have been applied to tissue culture studies. We have already seen how, for example, Watanabe and Raff (1988) used BrdU to label glial precursors in their study of cell fate in the retina.

There are, however, two means of following fate that are restricted almost exclusively to tissue culture. First, cells can be followed by direct observation. Second, cells can be cultured as individual cells so that their fate can be observed in total isolation from cell-cell contact.

The direct observation of developing cells is not impossible *in vivo*, but it is largely restricted to invertebrates and those rare vertebrates, such as zebrafish, whose embryos are small and transparent (Kimmel and Warga, 1988). In low-density culture, however, either with or without marker substances, cells can be directly observed with ease, especially with the aid of time-lapse microscopy. There are multiple examples of this in glial cell biology. The parameters that can be determined in this way include changes in morphological differentiation (Dubois-

Dalcq, 1987; Wolswijk and Noble, 1992), and rates and degree of symmetry of division (Wolswijk and Noble, 1989). Thus aspects of a cell's fate can be observed directly.

The culture of single cells is, in a sense, the ultimate cell fate experiment. In the absence of neighboring cells, the cell's environment is as isolated and controlled as can be achieved experimentally. As we have already discussed, such an experiment will tell us something of a cell's developmental potential. In particular, the cell's constitutive pathway of development is revealed (not necessarily the same as its presumptive fate); that is, the fate the cell will assume in the absence of any further environmental influence. The O-2A cell provides a good example of this: grown alone in the absence of any factors except those required to keep the cell alive and dividing, O-2A cells generate oligodendrocytes (Temple and Raff, 1985, 1986). This, therefore, is considered to be the constitutive pathway of O-2A development (Raff, 1989). In this case, this is probably also this cell's presumptive fate. Most of the current data available suggests that O-2A cells generate predominantly (possibly exclusively) oligodendrocytes *in vivo* (for reviews see Raff, 1989; Goldman and Vaysse, 1991; B. Williams and Price, 1992).

It is clear from the foregoing discussion that there is in a sense a compromise in all studies of cell fate. At one extreme is the study of cell lineage. This is unique in that a cell only has one presumptive fate, and that is what the study of cell lineage reveals. There is a purity to such a study, but its interpretation is often difficult. Do cells take on different presumptive fates because of intrinsic differences in developmental potential, or because of environmental differences? Answers to such questions are seldom forthcoming from cell lineage studies. Questions of developmental potential require experimental interference, and two elements of compromise immediately arise: first, are cell populations, which are easier to study, representative of the single cells of which they are composed; second, how normal does a cell have to be, and how normal does its environment have to be to give results that are relevant to the *in vivo* situation, yet sufficiently simplified to be under experimental control? Such considerations are complex, and each experimental situation has to be considered on its own merits. The central position of cell lineage in this discussion is that it provides the control for the interpretation of experimental investigations. Cell lineage information is, therefore, indispensable in understanding the process of cell fate allocation, but seldom sufficient in itself.

CASE STUDIES: A COMPARISON OF RETINA AND CEREBRAL CORTEX

In this chapter, I have discussed the sorts of approaches that have been applied to studies of cell fate, the types of data these approaches provide, and how these bodies of data relate to each other conceptually. Let us consider finally the answers that have resulted from the application of combinations of these approaches to the study of central nervous system development using as examples the retina and cerebral cortex. These examples are useful, first, because we have a reasonable grasp of the lineage relationships in both cases, but also because the patterns of cell lineage in each case look quite different.

Cell lineage in the retina has been extensively studied using retroviruses in rodents (Sun and Wekerle, 1986; Turner and Cepko, 1987), and by label injection in amphibians (Holt et al., 1988; Wetts and Fraser, 1988). These different data concur substantially: precursor cells labeled throughout the period of retinal development tend to be composed of multiple cell types. Even if precursor cells are labeled just prior to their final or penultimate division such that clones of between one and four cells predominate, these clones are often composed of multiple types. This indicates that retinal precursor cells are multipotential and suggests that they retain their multipotency as long as they continue to divide (Figure 7-3). This final conclusion has been questioned on the grounds that mixed clones occur less frequently than would be expected from such precursor cells if their fate were determined purely stochastically (R. Williams and Goldowitz, 1992a), but even so the multipotential model probably fits the data best (Price, 1993b).

The only exception to the above finding was the retinal astrocytes. This cell type was not included in the clones composed of the other retinal cell types. Müller glial cells, on the other hand, were included in these retinal clones (Figure 7-3B). Thus Müller cells are derived from the same population of precursor cells that generate the retinal photoreceptors and neurons, but the astrocytes are not. As we have already seen, this is consistent with culture data that suggest that the astrocytes are derived from progenitor cells that migrate into the retina from the optic nerve (Watanabe and Raff, 1988). Oligodendrocytes do not come into the equation at least in rodents because their retinas do not have this cell type. We have as yet no lineage information from species, such as the rabbit, that have retinal oligodendrocytes, but there is indirect evidence that, like astrocytes, these cells migrate into the retina (Schnitzer, 1985; ffrench-Constant et al., 1988).

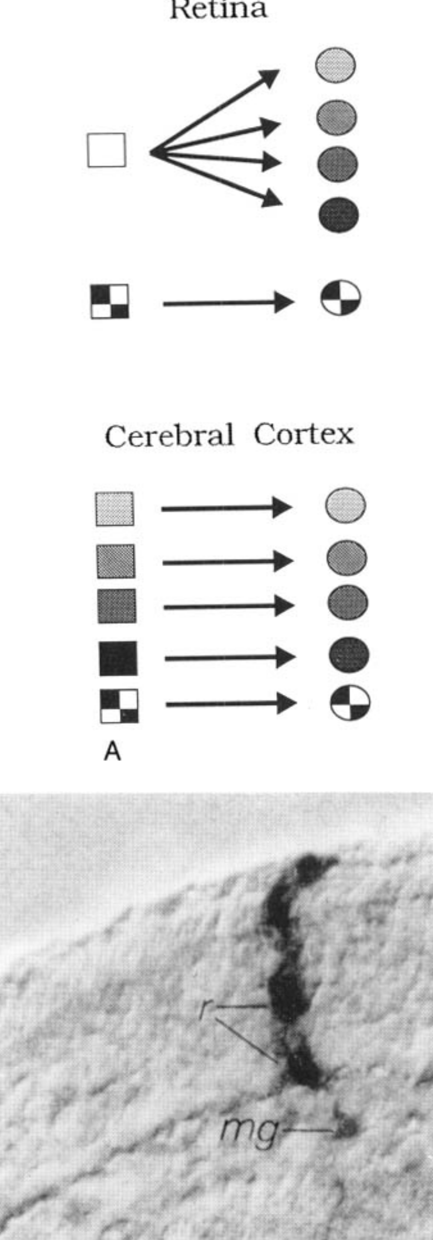

FIG. 7-3. (A) Lineage models in retina and cerebral cortex. The shaded ovals represent differentiated cells of different types. The rectangles represent their precursor cells. In the retina, a variety of different cell types are derived from a multipotential precursor cell (*unshaded rectangle*). Astrocytes (the chequered oval), however, are derived from a separate precursor cell population. In the cortex, each cell type seems to have its own precursor cell population. (B) Retinal Müller glia are derived from the same precursor cells as the retinal neurons and photoreceptors. The photomicrograph shows a retrovirally labeled clone in the retina. It is made up of a number of rod photoreceptors (*r*) and a Müller glial cell (*mg*). [From Turner and Cepko (1987), with permission.]

In the cerebral cortex, the picture looks very different (Figure 7-4). Studies with retroviral vectors in the rat embryo give clones that are composed almost entirely of a single cell type (Luskin et al., 1988; Price and Thurlow, 1988; Grove et al., 1993). Three, possibly four, of these are glial: there are distinct clones of gray matter astrocytes, white matter astrocytes, oligodendrocytes, and a fourth type of cell that appears astrocytic but does not contain glial fibrillary acidic protein (Grove et al., 1993). In addition, there are probably two types of neuronal clone (Parnavelas et al., 1992). So unlike the retina where precursor cells are multipotential, in the cortex they appear, at least by the time neurogenesis has commenced, to have become dedicated to the production to a single cell type.

There are probably some exceptions to this general rule. Several investigators have reported clones that are composed of multiple cell types (Price and Thurlow, 1988; Grove et al., 1993; Walsh and Cepko, 1992), but although all the data are not entirely consistent such clones appear at present to be relatively rare. More clearly demonstrated is a precursor cell, observed both *in vivo* and in cultures of cortical cells, that generates both neurons and oligodendrocytes (B. Williams et al., 1991). The significance of a cell with this slightly odd bipotentiality is not yet clear, but clones with mixed cell types probably represent multipotential precursor cells remaining from earlier developmental stages when, presumably, they were predominant. There are as yet no data on such early cortical precursor cells, although the forebrain, both in the embryo (Temple, 1989) and the adult (Reynolds et al., 1992) does seem to include precursor cells with the capacity to generate neurons and astrocytes in culture. Some cell lines derived from the embryonic forebrain show a similar potential when transplanted ectopically (Renfranz et al., 1991; Snyder et al., 1992). It is not clear why cells that generate this combination have not yet been found *in vivo*, but any of the reasons already discussed could explain the discrepancy. Also, it is possible that the lineage experiments, which are still incomplete, could have missed such clones.

There is a complication associated with these cortical data that does not apply to the retinal results. As already noted, a disadvantage with the retroviral approach is that the exact number and position of the infected cells is not known with certainty. Thus, if clones were to disperse too freely, there would be a possibility either that separate labeled clones could become superimposed so as to resemble a single clone, or that one clone could split into two halves, each of which were taken for a complete clone (Walsh and Cepko, 1992). Needless to say, either eventuality could confuse the lineage picture that emerged. In the retina, clones disperse to a very limited extent (Turner and Cepko, 1987), a finding that has been confirmed by the analysis of rodent chimeras (Goldowitz, 1989). In the cortex, however, the suggestion is that dispersion is considerable (Walsh and Cepko, 1988, 1992). Since the great majority of clones appear to have a single cell type, it is unlikely that they are becoming superimposed since this would tend to generate mixes. However, the possibility that two apparent clones could be one clone split in two must be considered.

The data *in vivo* are not yet sufficiently complete to resolve this issue, but studies in tissue culture suggest that the splitting of clones is not sufficient to

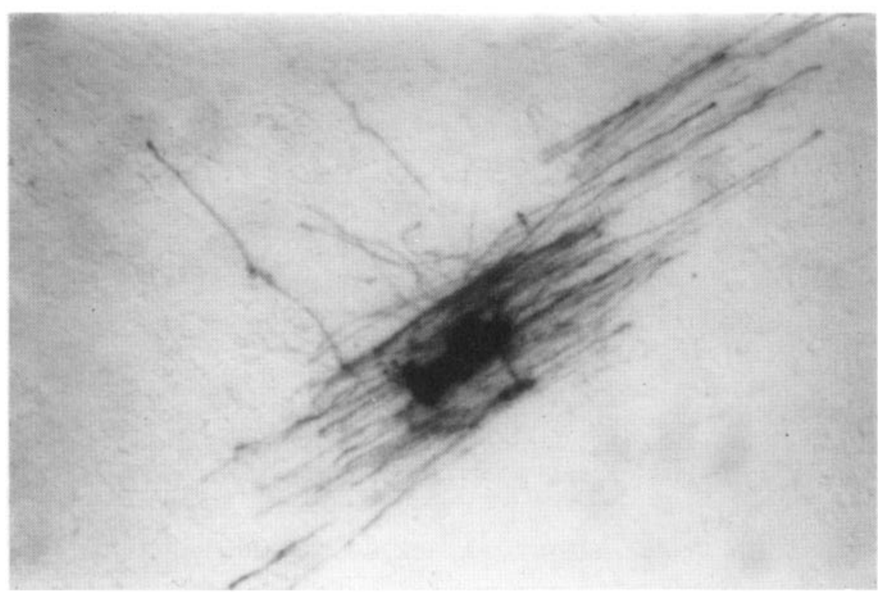

FIG. 7-4. A clone of oligodendrocytes in the external capsule. The precursor cell that gave rise to this cluster of oligodendrocytes was labeled by injecting a retrovirus into the cerebral vesicle at embryonic day 16 in the rat embryo as described in Grove et al. (1993).

distort the data appreciably. When cortical cells are labeled with virus and grown in culture, each clone only generates a single cell type, as *in vivo* (B. Williams et al., 1991). The exception is the neuron-oligodendrocyte combination already noted. The difference between studies of cells in culture and *in vivo* has been dwelt on sufficiently in this chapter with being repeated here. Nonetheless, the similarity between the two results suggests that if clones *in vivo* are being artefactually split into two parts, then both parts are probably composed of the same cell type.

The contrast between glial lineages in the retina and the cerebral cortex, therefore, could not be starker (Figure 7-3). In the former, there are no indigenous glial precursor cells as such; the only native glial cell, the Müller cell, comes from a multipotential precursor cell. In the cortex, there are at least three populations of dedicated glial precursor cells during the embryonic period, plus the N-O cell. The significance of cell lineage studies in defining questions in glial cell development could not be more dramatically outlined than by this comparison.

The challenges for the future lead in a number of directions. For those regions where the lineage pattern is becoming clear, the problem now is mechanism. In the retina, what determines the fate of the multipotential precursor cell? This question has both cellular and molecular aspects. Presumably cells influence each other in bringing about fate decisions. Indeed there is already evidence for such interactions (Reh and Tully, 1986; Watanabe and Raff, 1990). We also need to understand the factors that mediate such interactions, and how they work to change cell behavior (Anchan et al., 1991; Watanabe and Raff, 1992). In the cortex, we can ask what makes the different precursor cell types different, and what controls the behavior of each precursor. Fate determination, however, is still a problem in the cortex, since we have yet to discover when and in which cells fate becomes determined. The problem is that the cell lineage data is still incomplete.

In other regions, the study has yet to be begun. One of the intriguing questions is whether the retinal or cortical patterns of lineage will turn out to be the more typical. Currently, the retinal pattern seems to have the edge in that multipotential precursor cells seem to predominate in the optic tectum (Gray et al., 1988; Galileo et al., 1990; Gray and Sanes, 1992), the spinal cord (Leber et al., 1990), and the neural crest (Bronner-Fraser and Fraser, 1988, 1989) of the chick. On the other hand, the hindbrain seems to be composed of dedicated precursor cells from very early stages (Fraser et al., 1990). Since most of these studies have been on early chick embryos, much of glial lineage, which occurs later, is particularly obscure. Most of the questions surrounding glial cell lineage, however, now seem approachable. I would anticipate the next decade to be a very productive one in regard to this field of study.

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8 The origins and lineages of macroglial cells

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The question whether astrocytes, oligodendrocytes, and microglial cells are distinct types of cells in the brain was resolved only after an 80-year controversy. The debate began in the mid-nineteenth century and lasted until the 1920s when Rio Hortega showed with his classical silver impregnation methods that microglia are distinct from oligodendrocytes (Rio Hortega, 1921). Today, the existence of these three separate types of central nervous system glial cells is universally accepted, but their lineages and origins are still unclear. The questions asked by modern neurobiologists about glial cell lineage are the same types of questions as those asked over a century ago. Histologists in the late nineteenth century grappled with the question of whether neurons and glia are derived from a common stem cell (reviewed in Jones, 1929; Jacobson, 1991). One view, proposed by His in 1887, is that the germinal zone is composed of two populations of precursor cells, one giving rise to neurons and the other to glia. The opposite view, put forth by Schaper and others in the 1890s, is that the cells in the germinal zone are multipotential, capable of giving rise to both neurons and glia. The two positions are still being debated and, even using the latest techniques such as retroviral tracing, evidence can be found to support either His' or Schaper's hypothesis. The origins of microglia are equally uncertain, with one theory in support of a mesenchymal origin, while another proposes a neuroectodermal origin. The two theories have traded popularity over the past 70 years. In the 1980s, for example, considerable evidence accumulated in favor of a mesenchymal-macrophage origin for all microglia, but evidence has accumulated in this decade to support a neuroectodermal origin for some microglia (e.g., DeGroot, 1992; Chapter 10, this volume). If some microglia do originate from neuroectoderm, when does this cell type split away from the macroglial lineage and what neural cells are they most closely related to?

The critical issue about the lineage of macroglial cells concerns whether astrocytes and oligodendrocytes are derived from a common precursor cell or whether they derive from separate precursors. This issue is the subject of the present chapter, and our discussion focuses on the origins of macroglia in pre- and postnatal development and the lineage of macroglia in postnatal development. We will define the *origin* of glia as the region of the ventricular zone where glial precursors are born in relation to their final destination in the brain (e.g., the precursors of oligodendrocytes in the spinal cord may originate exclusively from the ventral half of the ventricular zone of the neural tube) (Warf et al., 1991). We will consider *lineage* in this chapter in a fairly restrictive sense and use the term to refer solely to a cell's *developmental history* or to its *ancestry* in the central nervous system. Using this definition, cell lineage is much like tracing our own family ancestry. The lineage of a cell may be determined without knowledge of *how* and *when* environmental factors interact with intrinsic, genetic determinants. It is studied by determining the phenotype of the precursor cell and the phenotype(s) of all its daughter cells. It thus defines the *normal* derivation of a group of cells within the central nervous system, without consideration of the factors that influence the lineage pathway taken by individual cells. Cell lineage is but one aspect of a larger picture defining a cell's developmental fate and potential. The concept of cellular committment and the ability of cells to adopt alternative fates (Jacobson, 1991; Chapter 7, this volume) are all properties of a cell's development, which are, in most cases, deduced from experimental manipulation of the cell or of its surrounding environment.

IN VIVO STUDIES

Origins of Macroglia in the Brain

The ventricular cells that surround the cerebral ventricles, the spinal canal, the optic canal and the optic cup are the site of origin for all the neurons and glia in the central nervous system. The ventricular zone

is a single pseudostratified columnar layer whose cells, with time, will migrate out of it and populate the embryonic subventricular, intermediate, and marginal zones (The Boulder Committee, 1970). It has been generally assumed that each region of the ventricular zone is intermixed with progenitor cells capable of generating all the varieties of neurons and glia that are located in the adjacent gray and white matter of the adult nervous system. However, a number of recent studies suggests that the ventricular layer may be fate-restricted with regard to the types of cells it is capable of generating. These studies provide information not only about the origin of specific subtypes of glia in the brain, but they also reveal new properties of glia: they show glial progenitors may use unique migration routes to reach their destination and they provide information about the time of divergence of glial lineages. Most importantly, as additional information is obtained about fate-restricted zones, these studies should clarify neuronal and glial lineages by showing that cells in specific regions of the brain are capable of generating only one or more types of cells.

In the spinal cord, the origin of oligodendrocytes may be restricted to the ventral half of the ventricular layer (Warf et al., 1991). By culturing cells from either E14 ventral or dorsal segments of the cord, the investigators show that the ventral segments, but not the dorsal segments, have the potential to generate oligodendrocytes. The dorsal half does not exhibit this capacity until E16, strongly suggesting that the precursors of oligodendrocytes migrate from the ventral to the dorsal half of the cord. Using Dil to label cells in the ventral half of the cord, Warf and collaborators found galactocerebroside $(GC)^+$, Dil cells in the dorsal half within 18 hours. Presumably, after the oligodendrocyte precursors migrate to the dorsal half, they divide and populate the sensory half of the cord.

In the retina, it is well established that precursor cells in the retinal neuroepithelium give rise to the different types of neurons and to the Müller cells, a modified form of astrocyte (e.g., Wetts and Fraser, 1988). The origin of the astrocytes in the nerve fiber layer of the retina is less clear but several lines of evidence suggest that the astrocyte precursors migrate into the eye. Retroviral studies (Turner and Cepko, 1987) and tissue culture work suggests that astrocytes migrate into the retina from the optic nerve (e.g., Stone and Dreher, 1987; Huxlin et al., 1992). These results suggest that the neuroepithelium of the retina is not competent or loses its potential very early in development to generate astrocytes in the nerve fiber layer.

In theory, the optic nerve should be capable of generating both neurons and macroglia. The anlage of the optic nerve is structurally similar to that of the rest of the neural tube in that it consists of a pseudostratified neuroepithelium surrounding the optic canal. The neuroepithelium is a heavily mitotic zone until the time it disappears around E16–17 (Skoff et al., 1976a). One line of evidence suggests that the nerve is intrinsically capable of generating both neurons and macroglia, while another line of studies suggests that oligodendrocytes and most astrocytes are derived from progenitors migrating into the nerve. Cultures prepared from mouse optic stalk early in development (Theilers stage 16-19) have the potential to generate both neurons and glia but the neuronal potential is quickly lost a half-day later (Juurlink and Fedoroff, 1980). The potential to generate neurons correlates inversely with the invasion of retinal axons into the optic nerve. Once retinal axons invade the optic nerve, the neuronal capacity is lost. This intriguing observation suggests that axons may have a direct effect upon the fate and/or survival of neuroepithelial cells in certain regions of the brain. The chicken optic nerve also has the capacity to generate neurons (Giess et al., 1990) but apparently not oligodendrocytes (Giess et al., 1992). It is unclear if this inability is due to the lack of a developmental signal in the optic nerve occurring early in development or is due to migration of oligodendrocyte progenitors from the brain. In support of an extrinsic source for oligodendrocytes in the nerve, evidence for migration of a progenitor cell (O-2A), which can generate in tissue culture both oligodendrocytes and a subtype of astrocyte (type 2) from the diencephalon into the nerve around E18 has been shown (Small et al., 1987).

The evidence for the hypothesis that the ventricular zone consists of fate-restricted sites, which are destined to give rise to different types of neurons and glia, continues to mount, but its confirmation requires additional research. How early in development such fate-restricted zones might develop and the physical size of such zones is unclear. Likewise, how cells in the neuroepithelium interact with each other to alter their fate is uncertain, but recent evidence suggests that neuroepithelial cell to neuroepithelial cell contact and the phase of their cell cycle are important factors in determining the fate of neurons in the neocortex (McConnell and Kaznowski, 1991). The subventricular zone has long been considered to be the site of origin for macroglia in postnatal development (Paterson et al., 1973). The subventricular zone generates primarily astrocytes and oligodendrocytes but may also have the potential to generate neurons, at least when these cells are placed into culture (Reynolds and Weiss, 1992; Lois and

Alvarez-Buylla, 1993). These cells populate the corpus callosum, central white matter tracts in the cerebrum, and in the gray matter (Levison and Goldman, 1993; Luskin et al., 1993). The extent of their migration from the subventricular zone and their fate may be temporally related as cells leaving the zone of neonates generate both astrocytes and oligodendrocytes, whereas cells migrating from ventricular zone at 2 weeks differentiate mostly into oligodendrocytes (Levison and Goldman, 1993). The migration of cells away from the subventricular zone into the surrounding neuropil seems fairly restricted with most of the movement occurring in the lateral plane and little in the anteroposterior directions (Levison et al., 1993). However, evidence also supports the idea that glial progenitor cells are capable of migrating over considerable distances from one region of the developing cerebrum to settle into another brain region (Price and Thurlow, 1988). This hypothesis is strongly supported by transplantation and tissue culture studies that demonstrate migration of immature glia over long distances (e.g., Lachapelle et al., 1984).

The radial glial cell is generally recognized as the first subtype of glia to appear in the brain (Misson et al., 1991). This bipolar cell is present when neurons are being generated and migrating into the mantle layer. Depending on the species, radial glia may or may not be glial fibrillary acidic protein$^+$ (GFAP) (Cameron and Rakic, 1991); however, they express several unique antigens which are not expressed by neuronal precursors and can be used to distinguish them from neuronal precursors (Misson et al., 1991). This cell type is now clearly recognized as the direct source for some astrocytes found in the gray and white matter (Misson et al., 1991), confirming the hypothesis of Ramón y Cajal (1955). Ramón y Cajal's hypothesis was based upon static Golgi impregnations of glia at various early stages of development; more recent studies have used direct methods to show their transformation into astrocytes in the cerebrum and in the spinal cord (Voigt, 1989; Culican et al., 1990). Certainly, radial glia give rise to astrocytes in both the gray and white matter of brain and cord, but can they generate oligodendrocytes as well? This idea is not novel and was proposed by Penfield in the 1920s (Penfield, 1924). Recent publications continue to present circumstantial evidence that radial glia in certain regions of the brain can generate oligodendrocytes (e.g., Choi and Kim, 1984; Hirano and Goldman, 1988). The Warf study (1991), which shows migration of oligodendrocyte precursors out of the ventral half of the cord, occurs when apparently only radial glia are present; this research, along with the histological studies, supports the idea for an oligodendrocyte origin from radial glia. If further research confirms this hypothesis, then our concept of this cell subtype as being solely within the astrocyte lineage may be incorrect. The phenotype of the radial glial cell is certainly distinctive but may bear no relevance as to the type of the cells it is capable of generating. Future research may show that there are subpopulations of radial glia that are capable of generating only astrocytes, only oligodendrocytes, and, still others, both. This discussion serves to highlight the idea that, while we have made considerable progress in understanding the time of origin of radial glia in the brain, their role in neural migration and the ability of some radial glia to form astrocytes, much is still unknown about their developmental potential.

In the brain, immunocytochemical staining of embryonic rat cerebrum suggests that the ventricular zone consists of separate precursors for astrocytes and oligodendrocytes (LeVine and Goldman, 1988a, 1988b). At E16, two populations of cells can be detected in the ventricular zone, one cell population is vimentin$^+$ with the morphology of radial glia and another population is GD3$^+$, carbonic anhydrase$^+$, and iron$^+$. These latter cells are large and round with thick processes. Analysis of transitional forms suggest that the vimentin$^+$ cells turn into astrocytes, whereas the other cells contribute to the oligodendrocyte lineage. *In situ,* GD3 is expressed by cells which appear to be solely in the oligodendrocyte lineage but it is also expressed by subpopulations of neurons and it can be argued that it is not a unique cell type marker. In support of the hypothesis that the oligodendrocyte lineage is established very early in development, message for a specific myelin transcript (DM20) has been found in the rodent cerebrum around E15 (Timsit et al., 1992). These studies show that subpopulations of glial cells can be detected immunocytochemically or with *in situ* hybridization methods within the cerebral neuroepithelium in midembryonic development. While caution must be observed in drawing conclusions about cell lineages from these studies, the data clearly show some cells in the astrocyte and oligodendrocyte lineage diverge quite early in embryonic development.

With time, proliferation in the ventricular zone comes to a halt and this pseudostratified layer changes into a single-cell layer termed *ependyma.* The subventricular zone, beneath the ventricular layer, becomes the proliferative zone for macroglia in postnatal life and even in adults (e.g., Paterson et al., 1973). This layer remains quite large in certain regions of the adult brain and is thought to be a slowly proliferating zone capable of giving rise to

both astrocytes and oligodendrocytes (e.g., Smart, 1961; and see discussion above).

Lineages of Macroglia in Postnatal Development

At issue is the question of whether there are separate precursors for astrocytes and oligodendrocytes in the brain or whether a bipotential cell exists that generates both astrocytes and oligodendrocytes. The answer is extremely complex because it involves knowledge of *when* lineages diverge in the brain, clarification of the distinction between what cell lineage is *in situ* versus a cells' potential to alter its lineage when the normal external environment is changed, and it even hinges on the definition of astrocytes and oligodendrocytes (Skoff and Knapp, 1991a). Evidence from *in situ* studies and extrapolation of *in vitro* data to the animal suggests that both situations occur *in situ*. However, the extent to which data from tissue culture can be extrapolated to the *in situ* condition must be cautiously interpreted in terms of cell lineage. Information gained from tissue culture often involves experimental manipulation of cells, and it is easy to confuse cell lineage (ancestry) with the developmental potential of a cell. We use the term developmental potential or plasticity to refer to a cell's capacity to alter its *normal* developmental path (see end of this chapter). It is possible to know the lineage of a cell type without knowing whether environmental factors or what mechanisms direct the fate of a particular cell type. Of course, most lineage studies provide information not only about lineage *per se* but also other subproperties about lineage (e.g., whether it is determinate or indeterminate) (Turner et al., 1990; Jacobson, 1991).

Two approaches have been used to study glial lineages; the first we describe as an "anterograde" method and involves the tracing of glial lineages forward from ventricular cells to mature glia in young adults; the second we refer to as a "retrograde" method and involves trying to trace lineages backwards from mature cell to ventricular cell. In both approaches, similar or different methodologies may be used but retroviral labeling or injection of tracer dyes are associated with the "anterograde" approach. *In vivo,* these methods have been extremely helpful in delineating neuronal lineages beginning with the work of Sanes et al. (1986). Several studies have described clonal lineages for neurons and astrocytes or for neurons and oligodendrocytes (e.g., Galileo et al., 1990; Williams et al., 1991; Levison and Goldman, 1993) while another study indicates that neurons, astrocytes and oligodendrocytes are derived from separate progenitor cells in the cerebral cortex (Luskin et al., 1993). The results from these studies should not necessarily be viewed as contradictory for the studies were done at different times and in different locations of the brain and, most significantly, it is likely that clonal potentiality is quite diverse throughout the brain. Only recently have we begun to obtain information about macroglial lineages (Levison and Goldman, 1993; Luskin et al., 1993). Why have we learned so much about neuronal lineages using retroviral methodology and so little about glial lineages *in situ* within the last five years? A prerequisite for such studies is the ability to correctly identify all the clonal progeny of a single, labeled progenitor cell. Neuronal precursors complete their division in a relatively short span and their progeny differentiate relatively slowly into morphologically distinct subsets of neurons. When these cells are visualized with beta-galactosidase stain, they are easily identified by their distinctive shape and position in cortical layers or in spinal cord laminae. In contrast, division of glial precursors is protracted throughout development and precursors persist even in adult animals. Identification of all glia in a clone may be difficult because glial precursors or immature glia have very little cytoplasm and may not express adequate levels of astrocyte or oligodendrocyte specific markers to be detected with immunocytochemistry. Even identification of mature glia can be difficult if the conditions of tissue preparation do not adequately preserve the morphology and antigenic characteristics of the cell. In the study by Luskin and McDermott (1994) electron microscopy was utilized to visualize the beta-galactosidase reaction product within each cell and to definitively characterize all the cells within a clone. In this study, discrete clones were found for astrocytes and oligodendrocytes. In the study by Levison and Goldman, about 15% of the clones were mixed. The differences in results between the two studies could be due to number of clones analyzed (Luskin and McDermott, 1994) or in the identification of discrete clones (Levison and Goldman, 1993).

Although clonal analysis of glial lineages *in situ* is relatively limited, several studies have examined glial lineages in cultures by clonal analysis with retroviruses (Vassye and Goldman, 1990; Lubetski et al., 1992; Levison and Goldman, 1993) or by single-cell dispersion (Carnow et al., 1991). These studies show that the vast majority of clones (>97%) give rise to either astrocytic or oligodendrocytic colonies and 3% or less give rise to mixed colonies. Lubetzki et al. (1992) found mixed clones only when the medium is changed from serum-free to serum-containing media. The findings of the retroviral studies that most clones

generate either astrocytes or oligodendrocyte mimics the results of the cell dispersion study by Carnow and collaborators (1991). These mutual observations suggests that the retrovirus is randomly taken up by the different progenitor cells in these neonatal cultures, and retrovirally labeling is a legitimate tool for cell lineage analysis. These studies have important implications regarding not only for cell lineage *in situ* but also *in vitro* because they show that the vast majority of astrocytes and oligodendrocytes found in these cultures are derived from clonal cells directed to one or the other macroglial lineage. If the data from the culture studies is representative of the *in situ* condition, then this implies that a common progenitor cell is not a major source for macroglia generated in postnatal development. However, the studies also strongly suggest that a progenitor cell exists that is capable of generating both astrocytes and oligodendrocytes. The phenotype of this clonal cell is unknown from these studies, but in terms of the cell's commitment to a specific lineage, the two most likely possibilities is a cell that is uncommitted and bipotential or it may be a cell that is directed along the oligodendrocyte lineage and is shifted back toward a bipotential stage (see discussion below).

A "retrograde" approach to the study of glial lineages has been used in a number of classical studies. With the development of good fixatives and perfusion methods in the early 1970s, electron microscopy or electron microscopy combined with thymidine autoradiography was used to study the morphology of immature glia (reviewed in Wood and Bunge, 1984). Fine structural analysis of proliferating cells in postnatal development revealed that astrocytes and oligodendrocytes in the early stages of differentiation are capable of dividing (Mori and Leblond, 1969, 1970; Skoff et al., 1976a). In the postnatal optic nerve, the vast majority (>80%) of 1 hour-pulsed thymidine-labeled cells have the phenotype of astroblasts or oligodendroblasts (Figures 8-1 and 8-2) (Skoff et al., 1976a). The oligodendrocytes undergo a gradual phenotypic transition from a proliferating "light" phase" to a nonproliferating "medium" and "dark" subtype characteristic of the mature, myelinating cell (Imamoto et al., 1978). These studies were interpreted to mean that the lineage for astrocytes and oligodendrocytes have already diverged postnatally and that most macroglia are generated directly from proliferation of their own cell type. Because all the proliferating cells cannot be identified in these preparations, it does not eliminate the possibility that a common progenitor cell divides and functions as a stem cell to produce more progenitors, which, in turn, generate astroblasts and oligodendroblasts. These early studies did not utilize immunocytochemical markers to identify the cells so their classification as astrocytes and oligodendrocytes has been questioned. More recently, the expression of oligodendrocyte and astrocyte specific markers has been studied in proliferating cells during postnatal development. The majority of proliferating cells in optic nerve or in presumptive white matter tracts of the brain express markers of committed cells at the time when the majority of glia are being generated (Skoff, 1990; Skoff and Knapp, 1991b; Skoff et al., 1994). During the first postnatal week in the optic nerve when the majority of astrocytes are generated, up to 40% of the proliferating glia in the optic system are GFAP$^+$ (Skoff, 1990). During the second postnatal week, when the majority of oligodendrocytes are generated, 30 to 100% of the dividing cells in presumptive white matter tracts immunostain with an oligodendrocyte specific antibody (AOO7) and a smaller percentage (10 to 15%) with another, slightly later-appearing oligodendrocyte specific antibody (RmAB) (Skoff and Knapp, 1991b; Skoff et al., 1994). The phenotype of the GFAP$^+$ astroblasts and the glycolipid$^+$ oligodendroblasts are quite different from each another, with the immature astroblasts having coarse, tapering processes and the oligodendroblasts having very long, thin processes. Morphologically, these immunostained cells match closely in size and cytoarchitecture those seen with the electron microscopic autoradiographic studies. These results imply that the vast majority of proliferating cells are already in one or another of the macroglial lineages. In viewing lineage in terms of cell ancestry, then, the immediate precursors for most astrocytes and oligodendrocytes are cells already within their own lineage (Figure 8-3). These findings do not negate the existence of a bipotential cell in postnatal development nor do they address the issue of when the astrocyte and oligodendrocyte lineages diverge prenatally. Taken together, the *in situ* prenatal and postnatal studies as well as the retroviral studies support the hypothesis that there are separate lineages for astrocytes and oligodendrocytes in postnatal development.

Role of O-2A Cells in the Postnatal Brain

In contrast to the hypothesis that the lineages for astrocytes and oligodendrocytes have diverged in postnatal rodent development, the hypothesis that a bipotential glial progenitor is the postnatal source for oligodendrocytes and type 2 astrocytes was proposed in the past decade (Raff et al., 1983a). Oligodendrocytes are thought to "develop from dividing progenitor cells" in the first postnatal week and

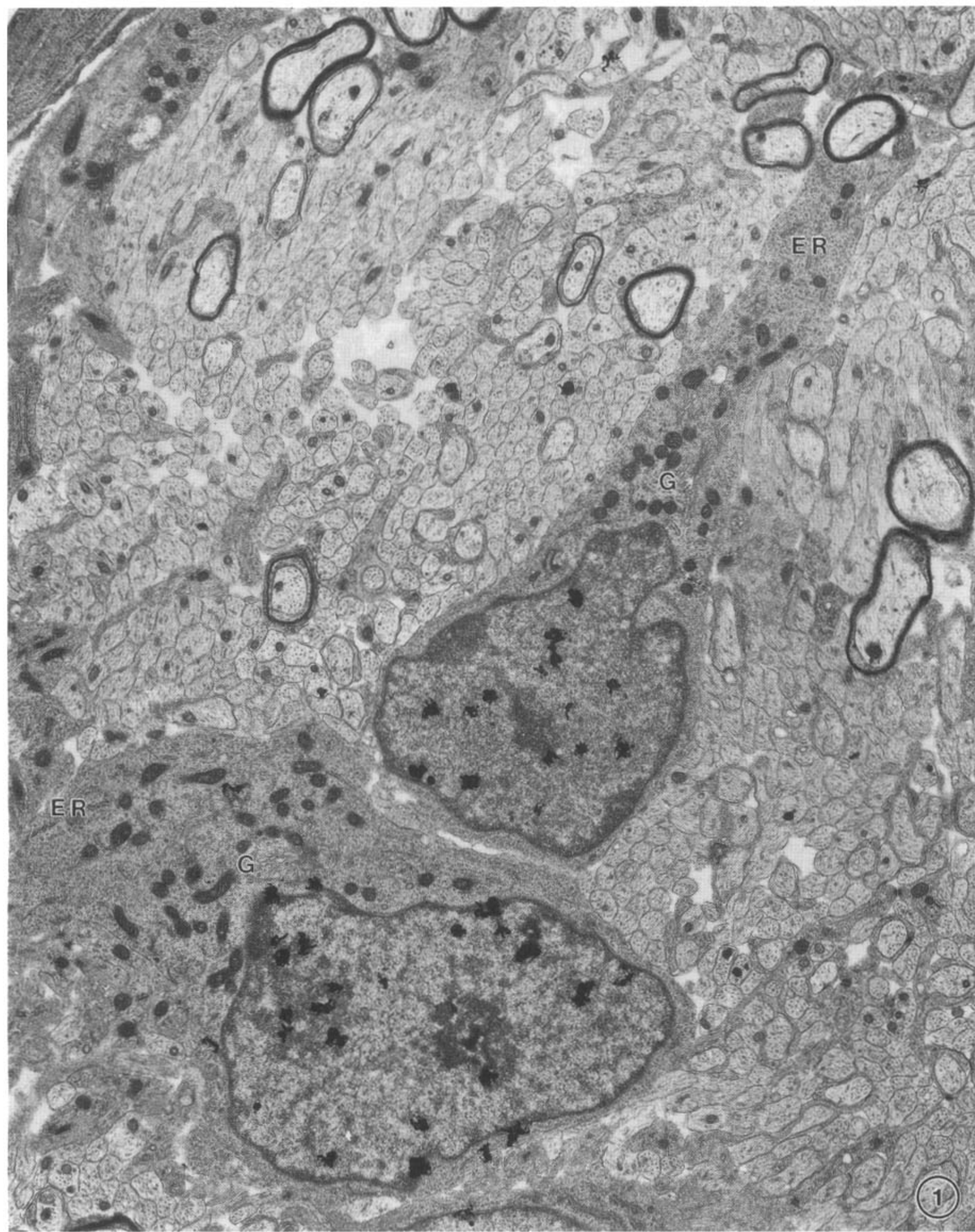

FIG. 8-1. This low-power electron microscopic autoradiogram is from the optic nerve of a 10-day-old postnatal rat. The rat was injected with tritiated thymidine 1 hour before sacrifice. Silver grains overlie the nuclei of two cells, indicating that these cells are in the S phase of the cell cycle. The cells exhibit the characteristic features of immature oligodendrocytes. Contrast them with the immature astrocyte in Figure 8-2. The cytoplasm of these oligodendroblasts is electron-dense and packed with free ribosomes, small mitochondria, Golgi apparatus (*G*), and rough endoplasmic reticulum (*ER*). The endoplasmic reticulum in these young oligodendrocytes is of narrow caliber and irregularly studded with ribosomes. The rough endoplasmic reticulum in oligodendroblasts is usually not stacked and prominent as in mature forms but scattered throughout the cytoplasm. A characteristic feature of oligodendroblasts is that the nucleus is situated at one end of the pole and the cytoplasm at the other end. The process of the oligodendroblast appears to be in contact with a myelin sheath, but it cannot be determined from this section. The cisternae of the Golgi apparatus (*G*) are narrow and closely apposed one on top of the other. Additional morphologic features distinguishing immature oligodendrocytes from astrocytes are detailed in Skoff et al. (1976a).

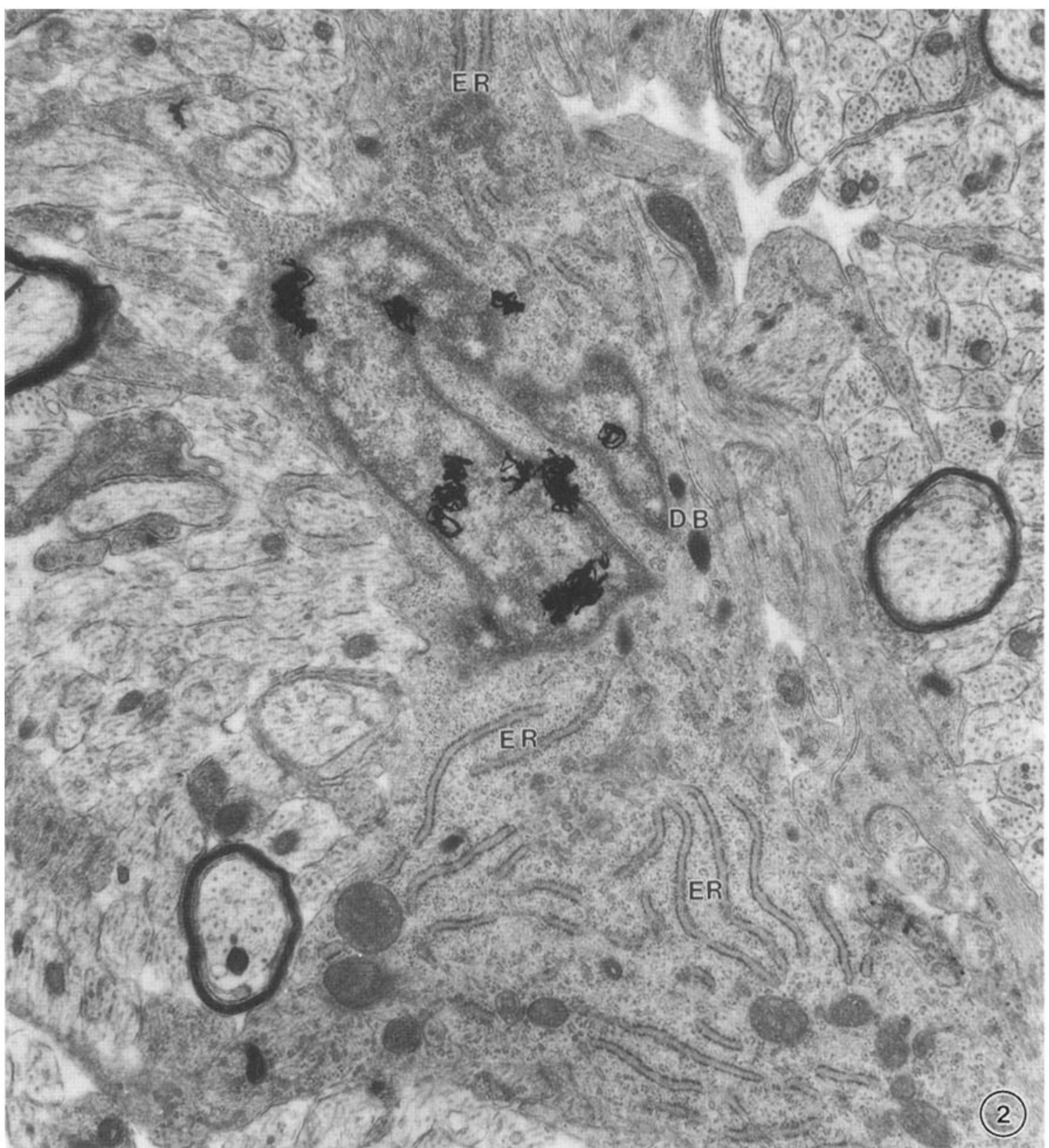

FIG. 8-2. This electron microscopic autoradiogram illustrates an immature astrocyte in the S phase of the cell cycle. Silver grains overlie the nucleus of this cell, which has been grazed near its edge. Such irregular contours are typical of astrocytes. Another view of this same cell is shown in Figure 6 of Skoff et al., 1976a. The wide-bore cisternae of the rough endoplasmic reticulum (*ER*) is filled with a floccular material and its membranes are evenly studded with ribosomes. Note how prominent the ER is in this cell compared to that in the oligodendroblasts in Figure 8-1. Numerous dense bodies (*DB*) are also frequently found in immature astrocytes but rarely in oligodendrocytes. Note also the irregular contours of the astrocytes' cytoplasm and how it radiates away from the nucleus in several directions. The paucity of mitochondria and free ribosomes in astrocytes is in sharp contrast to that of oligodendrocytes. It should be mentioned that immature astrocytes have few or lack intermediate filaments at this stage in their developnment.

type 2 astrocytes are thought "to develop from dividing progenitor cells beginning in the second week" (Raff, 1989). This bipotential O-2A cell is, like its *in vitro* counterpart, uncommitted until around the time of cell division, at which time growth factors (platelet-derived growth factor and ciliary neuronotrophic factor) drive them into becoming type 2 astrocytes or oligodendrocytes. The properties of these cells in culture is discussed below.

Since large numbers of O-2A cells are readily de-

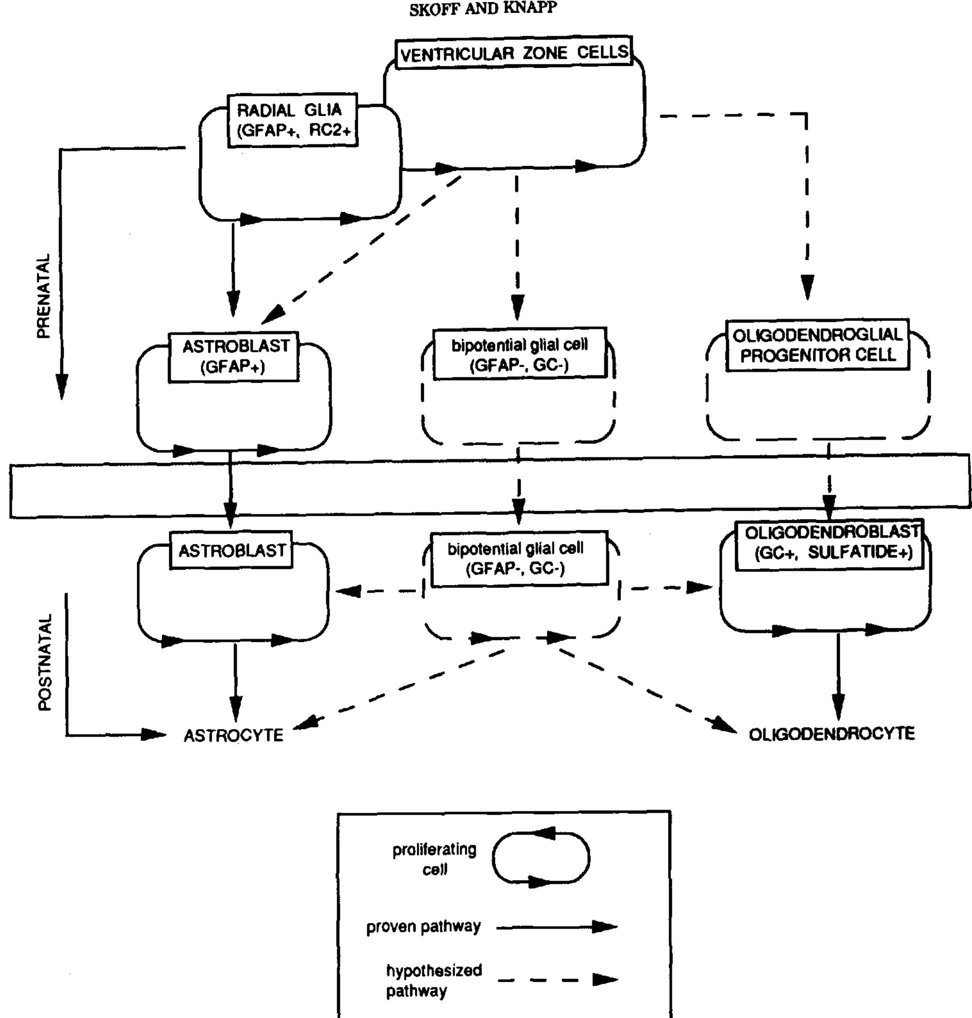

FIG. 8-3. This diagram depicts the scheme of gliogenesis in rodent brain combining information from many *in situ* studies. Solid lines indicate pathways for which evidence exists from *in situ* studies, while the dotted lines represent pathways for which there is less evidence. The pathways represent a simplified scheme of gliogenesis for all astrocytes and all oligodendrocytes. Future studies may reveal additional pathways for the subtypes of astrocytes and oligodendrocytes. [From Skoff and Knapp, (1991b), with permission.]

tected in tissue culture studies, they should also be detectable *in situ* if they play a major role in macroglial development. Studies with the A2B5 antibody *in vivo* have not reliably detected O-2A cells in tissue sections, partly perhaps because of the strong reactivity of the antibody with intracellular epitopes in neurons, which may mask surface staining on the glial cells (Eisenbarth et al., 1979; Fredman et al., 1984; Miller et al., 1989a). This staining has prevented the identification of the O-2A phenotype with this antibody in cells during development *in situ.* Since O-2A cells have a high rate of proliferation compared to GC^+ oligodendrocytes or $GFAP^+$ type 2 astrocytes in culture, it has been suggested that one contribution of O-2A cells during development might be as a source for new glial cells during expansion of the glial population. However, it should also be noted that $GFAP^+$ astrocytes and $glycolipid^+$ or MBP^+ oligodendrocytes *can* divide *in vitro.* In one study, 25% of the $GFAP^+/A2B5^+$ type 2 astrocytes grown in serum-containing medium incorporated [^{3}H]thymidine after continuous exposure for a couple of days (Raff et al., 1983a). Our results have shown that 1 to 2% of AOO7 or $R\text{-}mAB^+$ cells, and 1 to 3% of MBP^+ cells grown in serum-containing medium take up the proliferation marker bromodeoxyuridine after 1- or 18-hour pulses, respectively (Knapp, 1991; Knapp and Skoff, 1993). O-2A cells are therefore not the sole source of newly generated glia, even in cultures.

The common lineage for the progeny of the O-2A cells suggests they share a common function, and it is hypothesized that both are specialized for myelination (ffrench-Constant and Raff, 1986). The role of the oligodendrocytes in myelin formation is clearly defined, but the function of the "type 2" astrocyte is more circumstantial, owing to the problem of identifying $A2B5^+/GFAP^+$ cells *in situ,* as discussed above. Morphological studies in the optic nerve described a subpopulation of $GFAP^+$ cells with cell bodies located far from the pial surface whose fine, unbranching processes are longitudinally ori-

ented, running parallel to axons (Miller et al., 1989b). The processes of these astrocytes abut nodes of Ranvier and do not form endfeet on blood vessels or on the pial surface (ffrench-Constant and Raff, 1986). These astrocytes are suggested to be the *in vivo* correlates of the type 2 astrocyte (ffrench-Constant and Raff, 1986; Miller et al., 1989a, 1989b) described in culture since their time of origin and staining with J1 and NSP antibodies correlates with the staining and origin of type 2 astrocytes *in vitro*. This idea is appealing, since it suggests that both types of cells derived from O-2A progenitors are functionally involved with the process of axonal ensheathement and conductance of axon potential along a nerve. A study in which single glia are intracellularly filled with Lucifer Yellow, astrocytes fitting this description are not found (Butt and Ransom, 1989). Instead, all the astrocytes examined in this study have one or more processes that contact the pial surface regardless of the orientation of their processes (Butt and Ransom, 1989). Suarez and Raff (1989) and Sims and Gilmore (1991) observed that some astrocytes whose processes abut the pia also contact nodes of Ranvier. These findings contradict the idea that nodal association falls to a specific type of astrocyte whose cell body lies in the more interior portions of the nerve (ffrench-Constant and Raff, 1986; Miller et al., 1989a, 1989b).

The type 2 astrocytes in the optic nerve are described as being generated in a second wave after oligodendrocyte formation (Miller et al., 1985). Based upon quantitative immunohistochemistry at the light microscopic level, they should constitute the majority of the astrocytes in the optic nerve. However, a quantitative autoradiographic study of glial proliferation failed to find evidence for a second wave of astrocyte formation (Skoff, 1990). Cell proliferation in the rat optic nerve after 14 days postnatal drops dramatically and the few cells generated after the second postnatal week are typical oligodendrocytes when viewed ultrastructurally. If type 2 astrocytes are present in the nerve, they would have to be generated in a single wave before and overlapping with oligodendrocyte formation. Since different growth factors (platelet-derived growth factor and ciliary neuronotrophic factor) are thought to instruct O-2A cells into becoming oligodendrocytes or type 2 astrocytes *in situ,* this means that both of these growth factors need to be present at the same time in the optic nerve as oligodendrocytes and type 2 astrocytes are generated. If these growth factors are present at the same time and distributed fairly randomly throughout the nerve, it is unclear how O-2A progenitors could distinguish between them, divide, and their progeny select to enter either the oligodendrocyte or astrocyte lineage.

TISSUE CULTURE STUDIES

Although the preponderance of evidence suggests that oligodendrocytes and astrocytes *in situ* derive from lineages that are separate during postnatal development, the picture that has emerged from tissue culture studies is strikingly different. A study in the early 1980s suggested that there is a progenitor cell in dispersed cultures derived from postnatal central nervous system tissues that can develop into an oligodendrocyte or into a type of astrocyte, depending on the culture conditions (Raff et al., 1983a). When these bipotential, O-2A progenitor cells are cultured in serum-free medium they develop largely into oligodendrocytes, suggesting that this is the constitutive pathway. However, factors in serum apparently deter O-2A cells from their normal developmental pathway, causing them instead to develop largely into process-bearing, $GFAP^+$ astrocytes, which have been termed "type 2" astrocytes (Raff et al., 1983a). According to the O-2A theory, the flat, "type 1" astrocytes belong to a separate lineage and have not been observed to transform into process-bearing type 2 astrocytes under culture conditions so far examined (Raff et al., 1983b). Thus, in terms of lineage relationships, the O-2A theory suggests that type 2 astrocytes are more closely related to oligodendrocytes than they are to type 1 astrocytes. Although the original O-2A studies were done exclusively on cultures derived from optic nerve, similar observations have been made in cultures derived from cerebellum (Levi et al., 1986; Trotter and Schachner, 1989) and cortex (Trotter and Schachner, 1989).

Properties of O-2A Cells

The O-2A cell was initially identified by its reactivity with the A2B5 monoclonal antibody (Eisenbarth et al., 1979; Raff et al., 1983a). In typical experiments, when cells are grown in serum-containing medium, most cells that immunostain for A2B5 are also $GFAP^+$ process-bearing astrocytes but when grown in the absence of serum, most A2B5 cells are GC^+ (Raff et al., 1983a). It was concluded that a bipotential cell with A2B5 reactivity gives rise to the majority of type 2 astrocytes and oligodendrocytes within the cultures. In other studies, cells are initially exposed to A2B5 and then cultured for several days. Over 90% of the GC^+ oligodendrocytes that subsequently developed under serum-free conditions have residual A2B5 staining on their surface, suggesting

that a population of A2B5$^+$ cells are the direct precursors of oligodendrocytes in the cultures (Raff et al., 1983a). Further studies to characterize these O-2A cells with regards to their antigenicity and phenotype show that the cells have a simple, bi- or tripolar morphology (Temple and Raff, 1986; Small et al., 1987), immunostain for vimentin (Raff et al., 1984), are highly motile (Small et al., 1987) and proliferate with cell cycle times of approximately 18 to 20 hours (Noble et al., 1988). They share some electrophysiological properties with neurons, including a neuronal form of voltage-dependent sodium channel, and non-NMDA glutamate-activated ion channels (Barres et al., 1990). The ion channel phenotypes of O-2A cells and their differentiated progeny are distinct from one another (Barres et al., 1990), and there are indications that channel phenotype can differ among glia derived from different parts of the central nervous system (Sontheimer et al., 1989). Soluble factor(s) secreted by type 1 astrocytes in the cultures increase motility and proliferation of O-2A cells (Noble and Murray, 1984), and these effects can be mimicked by platelet-derived growth factor (Noble et al., 1988), suggesting physiological control mechanisms for expansion of the progenitor population *in vivo*. Other factors shown to be mitogens for O-2A cells include glial maturation factor (Hunter and Bottenstein, 1990), factors secreted by neuronal cell lines (Hunter and Bottenstein, 1990), and both α- and β-fibroblast growth factors (Besnard et al., 1989; Bogler et al., 1990; McKinnon et al., 1990). Although platelet-derived growth factor induces O-2A proliferation, the effect is only short term, and in the continued presence of platelet-derived growth factor, O-2A cells eventually drop out of the cycle and differentiate into oligodendrocytes (Noble et al., 1988). Fibroblast growth factor blocks progenitor cell differentiation and promotes continued proliferation, while at the same time upregulating the synthesis of platelet-derived growth factor receptors. Exposure to platelet-derived growth factor then induces migration (Small et al., 1987; Bogler et al., 1990) and differentiation (McKinnon et al., 1990). In this way, platelet-derived growth factor and fibroblast growth factor might act together to coordinate the timely differentiation of O-2A cells. Precursors of O-2A cells that are A2B5$^-$ also apparently respond to platelet-derived growth factor with increased proliferation and enhanced generation of O-2A cells (Grinspan et al., 1990).

In the optic nerve studies, flat astrocytes are reported to be mostly A2B5$^-$ while process-bearing cells are A2B5$^+$. This suggests a possible lineage relationship among the process-bearing cells, which includes both oligodendrocytes and astrocytes with processes and their precursor, the small, undifferentiated cells. In several more recent studies, the A2B5 antibody has been shown to immunostain a sizeable subpopulation of cortical and cerebellar flat, bed-layer astrocytes (Drago et al., 1990; Knapp, 1991; Vaysse and Goldman, 1992), although such immunostaining was never observed in the optic nerve studies that defined the O-2A cell (Raff et al., 1983a, 1983b). A2B5 is now well known to react with a variety of antigens, including multiple gangliosides and sulfatide (Kundu et al., 1983; Fredman et al., 1984; Majocha et al., 1989). Thus, cell types that are not closely related to each other either phenotypically or by lineage can immunostain for A2B5 by virtue of its affinity for different antigens. Such is the case for certain neurons that are A2B5$^+$ (Eisenbarth, 1979; Fredman et al., 1984), which are not in the same lineage as glial cells. The expression of A2B5 also appears to be inducible by growth factors within culture medium (Drago et al., 1990). It is now clear that A2B5 is not a monospecific antibody but that it reacts with a variety of epitopes and cell types, and its reactivity can be modulated by culture conditions.

Switching Glial Lineages In Vitro

A major finding of the O-2A studies is that oligodendrocytes and astrocytes retain their bipotentiality even after beginning to express what are considered to be cell-type specific markers (Raff et al., 1983a; Raff et al., 1984; Temple and Raff, 1986). In examining large populations of cells, GC$^+$ oligodendrocytes initially grown in serum-free medium can be placed in serum-containing medium and will either become GC$^-$/GFAP$^+$ or express a mixed GC$^+$/GFAP$^+$ phenotype. Likewise, the reversal of GFAP$^+$, type 2 astrocytes into GC$^+$/GFAP$^-$ or GC$^+$/GFAP$^+$ cells can be accomplished by switching culture conditions from serum-containing to serum-free media. After initial expression of GC or GFAP, developmental pathways can be switched for either 24 hours (Raff et al., 1983a) or 48 hrs (Raff et al., 1984; Temple and Raff, 1986) and the switch does not require cell division (Temple and Raff, 1986). None of these studies followed the switching of a single cell from an astrocyte to an oligodendrocyte phenotype and vice versa but the cells of mixed phenotype are thought to provide ample evidence that cells can switch from one phenotype to the other.

The original identification of cells with mixed astrocyte and oligodendrocyte phenotype are made using an antibody produced by Dr. B. Ranscht (Ranscht et al., 1982), now termed R-mAb. R-mAb

was thought to be specific for GC, but has since been shown to react with additional high specificity to sulfatide, and with lesser affinity to seminolipid and psychosine (Bansal et al., 1989). Other studies identified $GFAP^+$ cells that react with antibodies O1 and O4 (Raff et al., 1983a; Trotter and Schachner, 1989), which have been described as being oligodendrocyte-specific. We have also observed up to 10% of cells in serum-containing cortical cultures that are $GFAP^+$ and have the morphology of astrocytes, but which stain for O4 or O1 as well as for GFAP (R. P. Skoff and P. E. Knapp, unpublished observations). The O4 and O1 antibodies react primarily with sulfatide and GC, respectively, but with lesser affinity to other lipids including psychosine, seminolipid, and mono- and digalactosyldiglyceride (Bansal et al., 1989). Additionally, O4 reacts with an unidentified antigen (POA), which appears very early in glial development, even before the synthesis of sulfatide is detected (Bansal et al., 1992). In light of these multiple specificities, the results suggesting that astrocytes and oligodendrocytes can switch lineage must be cautiously interpreted. An alternative interpretation of $R\text{-}mAb^+/GFAP^+$ or $O1^+/GFAP^+$ cells is that subpopulations of astrocytes grown *in vitro* normally express antigens that react with R-mAb and O1. These astrocytic antigens might be the same or might be completely different from the antigen(s) found on the surfaces of oligodendrocytes. Interestingly, when Raff et al. (1983a) examined freshly dissociated suspensions of optic nerve or corpus callosum, $GFAP^+/R\text{-}mAb^+$ cells are not found. This suggests that a mixed phenotype ($GFAP^+/R\text{-}mAb^+$, 01^+) is not a step in the normal *in vivo* differentiation of oligodendrocytes or astrocyte. Glia with a mixed antigenic phenotype ($GFAP^+/GC^+$) have been infrequently reported in brain tissues either during normal development (Choi and Kim, 1984) or after demyelination induced by antibodies to GC (Carroll et al., 1987). If GC is expressed by astrocytes in demyelinated tissues (Carroll et al., 1987), such expression may be unrelated to normal glial lineage and may instead represent a transient expression of an inducible antigen.

A UNIFYING THEORY OF GLIOGENESIS

The cell lineage studies presented above may appear contradictory but, when taken as a whole, the *in vivo* and *in vitro* findings can be interpreted as converging into a unifying theory of gliogenesis. The *in vivo* studies point to *separate* and *early divergence* of the astrocyte and oligodendrocyte lineages. The *in vitro* studies point to a *bipotential cell* in postnatal development, which generates both oligodendrocytes and a process-bearing type of astrocyte. How can these two lines of evidence be integrated? The evidence is very strong that some glial precursor is present in postnatal development which has the potential *in vitro* to generate both types of macroglia. However, this potential is apparently not expressed *in vivo* under *normal* developmental conditions. When O-2A cells are purified *in vitro,* loaded with the long-term dye fast blue, and then transplanted into neonatal rat brain, the fast blue-labeled cells develop exclusively into oligodendrocytes (Espinosa de los Monteros et al., 1993). In a similar vein, transplantation of O-2A progenitors into demyelinated brain leads to the formation of mature oligodendrocytes (Groves et al., 1993). The data can be interpreted two ways: the microenvironment of the developing brain restricts the bipotentiality of cells phenotyped as O-2A cells in culture or the O-2A cells are all cells "committed" to the oligodendrocyte lineage. Whichever is the case, the O-2A cells are probably somewhat less differentiated than the $glycolipid^+$ (RmAb, A007), multiple process-bearing cells that have been described in postnatal brain and referred to as oligodendroblasts (Skoff and Knapp, 1991b). Alternatively, they could be $glycolipid^+$ and at the same stage of differentiation as the oligodendroblasts described in the brain but lose these surface antigens and their fine processes when dissociated and placed in culture. Regardless of the exact degree of differentiation between the O-2A cell and the oligodendroblast, the fact that a progenitor cell in culture can develop into a subtype of astrocyte, as well as an oligodendrocyte, demonstrates that extrinsic factors can modulate this cell's potential to alter its fate. Conversely, the transplantation studies demonstrate that *in situ* conditions restrict the potential of cells phenotypically defined as O-2A cells *in vitro.* A major, unresolved question is whether all or only a certain percentage of cells phenotypically identified as O-2A cells in culture have the potential to switch lineages. The bipotential property of the O-2A cells has been examined in bulk populations of cells and in nondividing cells over a short time interval (Temple and Raff, 1986). Most convincingly, the bipotentiality of immature glial cells needs to be demonstrated by following single cells over extended periods of time to show that they divide and that both astrocytes and oligodendrocytes can be generated from one cell.

If the normal fate of O-2A cells within the brain is to generate oligodendrocytes, this implies, by default, that a separate lineage or lineages exist(s) for the various subtypes of astrocytes found throughout the nervous system. Evidence that such distinct as-

troglial lineage(s) exist is shown by the transformation of radial glia into astrocytes and the large number of proliferating, GFAP+ astrocytes found in postnatal brain at times when astrocytes are normally generated.

As has been described by Jacobson (1991), lineage in many invertebrate systems is determinant, meaning that cell fate is fixed regardless of the surrounding environment. In vertebrate systems, however, lineage is usually indeterminant, meaning that cell fate is dependent on extrinsic conditions and *probabilistic.* The controversy about macroglia lineages is an excellent case in point. Integrating the current *in vivo* and *in vitro* evidence, the *in vivo* counterparts of the *in vitro* O-2A cells will generate all oligodendrocytes (and not a subset of astrocytes) in the brain with very high probability. Because this probability is very high, proliferation and differentiation of O-2A cells into oligodendrocytes in the brain can be considered their *normal* path of development. Returning, then, to the definition of cell lineage in terms of a particular cell's ancestry, the ancestors of oligodendrocytes in the postnatal brain are in a lineage separate from astrocytes. However, the *indeterminancy* of the oligodendrocyte lineage can be demonstrated when cells in this lineage are removed from the brain. They are subject to a different set of extrinsic conditions than occur within the brain and their behavior *in vitro* deviates from their normal developmental course. They now display a *plasticity* that is very infrequently observed *in situ* in normal development, and this is reflected in the cell's ability to switch its normal fate and phenotype. The critical issue is not to equate a particular cell's lineage *in situ* with this cell's plasticity *in vitro.*

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9 *In vitro* studies of the development, maintenance and regeneration of the oligodendrocyte-type-2 astrocyte (O-2A) lineage in the adult central nervous system

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The physiological requirements for the generation of oligodendrocytes in the developing and mature central nervous system differ fundamentally. The millions of oligodendrocytes that are formed during development, together with many other differentiated cell types, participate in the formation of the complex mature central nervous system. In contrast, the generation of oligodendrocytes in the adult central nervous system has the primary purpose of maintaining homeostasis, that is, to compensate for naturally occurring oligodendrocyte cell death and to respond to demyelinating damage. Thus, in the adult central nervous system there is normally only a modest production of oligodendrocytes and large numbers of new oligodendrocytes are only generated following demyelination. This suggests that the division and differentiation of the precursor cells that give rise to oligodendrocytes in the developing and mature central nervous system may be controlled by different mechanisms. However, are these differences a reflection of differences in the *in vivo* environment of the oligodendrocyte precursor cells or are they a reflection of differences that are intrinsic to the precursor cells themselves?

Oligodendrocytes in the developing and mature central nervous system appear to be generated by two distinct precursor populations. This suggestion has come from *in vitro* studies of the oligodendrocyte-type-2 astrocyte (O-2A) lineage of the rat optic nerve, where *perinatal* and *adult* precursor cells are referred to as O-2A^{perinatal} and O-2A^{adult} progenitor cells, respectively (Wolswijk and Noble, 1989). Detailed comparisons of the properties of perinatal and adult optic nerve-derived O-2A progenitor cells have indicated that these two populations differ fundamentally in tissue culture (Wolswijk and Noble, 1989; Wren and Noble, 1989; Van der Maazen et al., 1991a; Wren et al., 1992; Borges et al., 1995) (see Table 9-1). At least some of the differences between O-2A^{perinatal} and O-2A^{adult} progenitor cells appear to be related to the differences in the physiological requirements of the developing and adult central nervous systems, respectively (Wolswijk and Noble, 1989).

Studies on O-2A^{perinatal} and O-2A^{adult} progenitor cells have also yielded new insights into (1) mechanisms that may be involved in halting the exponential increases in the number of oligodendrocytes seen during development, (2) the developmental origin of the stem cell-like O-2A^{adult} progenitor cells, (3) mechanisms that may underlie regenerative events in the oligodendrocyte lineage, and (4) factors that may hamper repair of demyelinated lesions.

COMPARATIVE PROPERTIES OF O-2A^{adult} AND O-2A^{perinatal} PROGENITOR CELLS GROWN *IN VITRO*

In this chapter, the properties of optic nerve-derived O-2A^{perinatal} and O-2A^{adult} progenitor cells are discussed. Although the O-2A lineage is also found in regions of the developing and adult central nervous system other than optic nerve (e.g., Levi et al., 1986; Behar et al., 1988; Trotter and Schachner, 1988; Gard and Pfeiffer, 1989; Armstrong et al., 1990; Vaysse and Goldman, 1990; Van der Maazen et al., 1991b; Warf et al., 1991; Barnett et al., 1993; Levine et al., 1993; G. Wolswijk, unpublished observations), O-2A progenitor cells derived from such regions have not been analyzed in as great detail as those isolated from perinatal and adult optic nerves. However, there are indications that O-2A progenitor

cells isolated from these regions may differ in some aspects from their optic nerve counterparts (e.g., Vaysse and Goldman, 1990; Van der Maazen et al., 1991b; Lubetzki et al., 1992; G. Wolswijk, unpublished observations).

O-2A progenitor cells isolated from the optic nerves of perinatal and adult rats can be induced to differentiate along two different developmental pathways. Growth of O-2A^{perinatal} and O-2A^{adult} progenitor cells in a chemically defined medium (DMEM-BS) is associated with their precocious differentiation into oligodendrocytes (Raff et al., 1983b; Temple and Raff, 1985; ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989; Chan et al., 1990), the cells that form an insulating sheath of myelin around axons in the central nervous system (Peters et al., 1976). O-2A^{perinatal} and O-2A^{adult} progenitor cells can also differentiate into type-2 astrocytes *in vitro,* but this requires the presence of an induction factor(s), such as a factor(s) found in fetal sera (Raff et al., 1983b; ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989; Lillien and Raff, 1990). It should be noted, however, that the *in vivo* existence of type-2 astrocytes is a matter of great controversy (e.g., Fulton et al., 1991; Noble, 1991; Skoff and Knapp, 1991; Chapter 8, this volume) and that the studies discussed in this review have focused primarily on the differentiation of O-2A^{perinatal} and O-2A^{adult} progenitor cells into oligodendrocytes.

When O-2A^{perinatal} and O-2A^{adult} progenitor cells are grown on monolayers of cortical astrocytes or in the presence of DMEM-BS conditioned by cortical astrocytes (Astro-CM) these cells retain their progenitor phenotype, that is, these cells are stimulated to divide and at the same time are prevented from differentiating prematurely into oligodendrocytes (Noble and Murray, 1984; Raff et al., 1985; ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989; Chan et al., 1990). Cortical astrocytes appear to be very similar to the type-1 astrocytes of the optic nerve (Raff et al., 1983a), suggesting that type-1 astrocyte-derived factor(s) may also be important in controlling the proliferation and differentiation of O-2A^{perinatal} and O-2A^{adult} progenitor cells *in vivo.* This notion is supported by observations that type-1 and cortical astrocyte-derived factor(s) are capable of supporting the division and correctly timed differentiation of embryonic O-2A^{perinatal} progenitor cells into oligodendrocytes *in vitro* (Raff et al., 1985, 1988).

In vitro studies have shown that O-2A^{adult} progenitor cells differ in many ways from their perinatal counterparts (see Table 9-1):

1. Freshly isolated O-2A^{perinatal} progenitor cells contain the intermediate filament protein vimentin (Raff et al., 1984; Wolswijk and Noble, 1989; Wolswijk et al., 1990), whereas virtually all O-2A^{adult} progenitor cells grown for 1 day *in vitro* lack intermediate filaments of any class (Wolswijk and Noble, 1989).
2. O-2A^{adult} progenitor cells have much longer cell cycle times than O-2A^{perinatal} progenitor cells when induced to divide by cortical astrocyte-derived factor(s), resulting in much slower increases in the number of O-2A lineage cells in cultures of adult optic nerve as compared to perinatal optic nerve cultures (Wolswijk and Noble, 1989).
3. When O-2A^{adult} and O-2A^{perinatal} progenitor cells are grown on cortical astrocytes or in Astro-CM, O-2A^{adult} progenitor cells express a pseudo-unipolar morphology with one major process plus several

TABLE 9-1. *Distinguishing Properties of O-2A^{perinatal} and O-2A^{adult} Progenitor Cells Grown* in vitro

Properties	O-2A^{perinatal} Progenitor Cell	O-2A^{adult} Progenitor Cell
Antigenic phenotype	O4$^-$Vimentin$^+$	O4$^+$Vimentin$^-$
Morphology	Bipolar	Unipolar
Cell cycle time	18 ± 4 h	65 ± 18 h
Rate of migration (on PLL)	21 ± 2 μm/h	4 ± 1 μm/h
Time-course of differentiation (50% differentiated)	<< 2 days	3–5 days
Developmental potential[a]	Tripotential	Bipotential
Mode of division and differentiation[a]	Symmetric	Asymmetric
Self-renewal capability[a]	Limited	Unlimited (?)
Sensitivity to complement in the absence of antibodies[b]	Insensitive	Sensitive
Sensitivity to irradiation[c]	Relatively sensitive	Relatively insensitive

Modified from Wolswijk and Noble (1989), with permission.
[a]Wren et al. (1992).
[b]Wren and Noble (1989).
[c]Van der Maazen et al. (1991a).
PLL, poly-L-lysine.

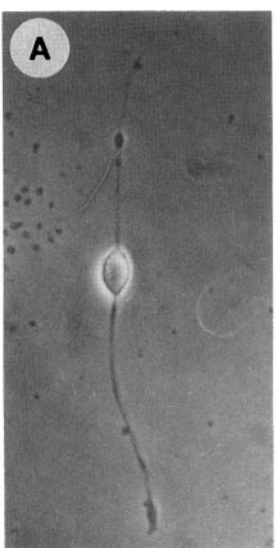
smaller and thinner processes (Wolswijk and Noble, 1989; Chan et al., 1990), while O-2A^{perinatal} progenitor cells have a characteristic bipolar morphology (Temple and Raff, 1986; Small et al., 1987) (see Figure 9-1).

4. O-2A^{adult} progenitor cells stimulated to divide by cortical astrocytes *in vitro* bind the O4 monoclonal antibody (Wolswijk and Noble, 1989), whereas proliferating O-2A^{perinatal} progenitor cells are O4$^-$ (I. Sommer and M. Noble, unpublished observations; Wolswijk and Noble, 1989; Wolswijk et al., 1990; Gard and Pfeiffer, 1990).

5. O-2A^{adult} progenitor cells migrate on average five times more slowly than their perinatal counterparts when cultured on a poly-L-lysine coated substrate in Astro-CM (Wolswijk and Noble, 1989).

6. The differentiation of O-2A^{adult} progenitor cells into oligodendrocytes or type 2 astrocytes *in vitro* takes about three times longer than is the case for O-2A^{perinatal} progenitor cells (Wolswijk and Noble, 1989).

Accumulating evidence indicates that O-2A^{perinatal} and O-2A^{adult} progenitor cells induced to divide by cortical astrocytes express their distinguishing properties in response to a single cortical astrocyte-derived factor, that is, platelet-derived growth factor (PDGF). Cortical astrocytes secrete PDGF *in vitro* and O-2A^{perinatal} and O-2A^{adult} progenitor cells grown in purified or recombinant PDGF express the same properties as those grown in the presence of cortical astrocyte-derived factor(s) (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988; Wolswijk et al., 1991b). In addition, antibodies to PDGF neutralize virtually all of the mitogenic effect in Astro-CM for O-2A^{perinatal} and O-2A^{adult} progenitor cells (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988; Wolswijk et al., 1991b). These results suggest that the intracellular responses generated following the binding of PDGF to its receptor differs in O-2A^{perinatal} and O-2A^{adult} progenitor cells, resulting in the expression of different patterns of cellular behavior. PDGF may also be important in controlling the behavior of O-2A^{perinatal} and O-2A^{adult} progenitor cells *in vivo*, as both PDGF and its mRNA are expressed in the developing and adult optic nerve (Richardson et al., 1988; Pringle et al., 1989).



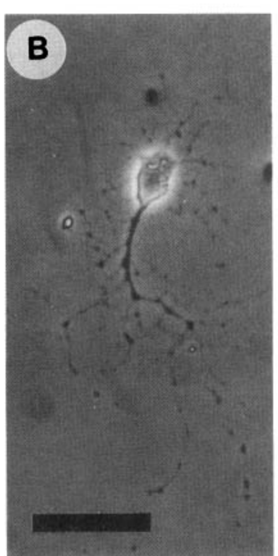


FIG. 9-1. O-2A^{perinatal} and O-2A^{adult} progenitor cells differ in their morphology when exposed to cortical astrocyte-derived factor(s) or platelet-derived growth factor. O-2A progenitor cells isolated from the optic nerves of newborn rats (A) and adult animals (B). Adult animals were grown for 3 days in Astro-CM and photographed, when still alive, through an inverted microscope. This figure illustrates that most O-2A^{perinatal} progenitor cells express a bipolar morphology *in vitro* (Temple and Raff, 1986; Small et al., 1987), while O-2A^{adult} progenitor cells predominantly have a pseudounipolar morphology (Wolswijk and Noble, 1989; Chan et al., 1990). Bar in Figure B = 50 μm.

APPEARANCE OF O-2A^{adult} PROGENITOR CELLS DURING DEVELOPMENT OF THE OPTIC NERVE AND THEIR ORIGIN

Developmental studies have suggested that the properties of O-2A progenitor cells do not gradually change with age, but that O-2A^{adult} progenitor cells appear in the developing optic nerve as a distinct population of cells and that these cells coexist with O-2A^{perinatal} progenitor cells until this latter population has disappeared from the nerve (Wolswijk et al., 1990) (see Figure 9-2). Cells that express properties of O-2A^{adult} progenitor cells in vitro can be isolated from developing optic nerves as early as postnatal day 7 (P7) (Wolswijk et al., 1990). Between the ages of P7 and P30, both O-2A^{adult} and O-2A^{perinatal} progenitor cells can be isolated, but the ratio of these two O-2A progenitor types gradually shifts in favor of O-2A^{adult} progenitor cells (Wolswijk et al., 1990). Cultures prepared from the optic nerves of adult rats only contain O-2A^{adult} progenitor cells (Wolswijk and Noble, 1989). These developmental studies have also shown that O-2A^{adult} and O-2A^{perinatal} progenitor cells express their distinguishing properties in the same culture (Wolswijk et al., 1990), thus providing direct evidence that O-2A^{adult} and O-2A^{perinatal} progenitor cells differ intrinsically from each other.

O-2A^{adult} progenitor cells appear to be derived directly from a subpopulation of O-2A^{perinatal} progenitor cells, as indicated by three separate findings. First, time-lapse cinemicroscopy studies have revealed that a subpopulation of cells with the char-

acteristics of O-2A^{perinatal} progenitor cells (i.e., cells with a bipolar morphology, short cell cycle time and high rate of migration) in cultures of P21 optic nerve grown in Astro-CM has the ability to generate unipolar, slowly dividing and migrating O-2A^{adult} progenitorlike cells (Wren et al., 1992). Second, serial passaging of populations of optic nerve cells derived from newborn rat pups [which lack O-2A^{adult} progenitorlike cells (Wolswijk et al., 1990)] onto fresh monolayers of cortical astrocytes is associated with the appearance of cells with the antigenic phenotype, morphology, and rate of division characteristic of O-2A^{adult} progenitor cells (Wren et al., 1992). After several passages, these cells become the dominant population of O-2A progenitor cells in the cultures. The O-2A^{adult} progenitor cells that appear in the cultures are generated by cells that are contained in the O-2A progenitor population at the start of the experiments, as no new O-2A lineage cells appear in the cultures when O-2A lineage cells are eliminated specifically from the cultures. Third, colony studies have suggested that some colonies of O-2A^{perinatal} progenitor cells have the ability to generate cells with some of the properties of O-2A^{adult} progenitor cells (Dubois-Dalcq, 1987; G. Wolswijk, unpublished observations). These findings are consistent with the possibility that O-2A^{adult} progenitor cells arise in the optic nerve itself and that these cells do not enter the developing optic nerve as a second population of O-2A progenitor cells.

THE MAINTENANCE OF THE O-2A^{adult} PROGENITOR POPULATION THROUGHOUT LIFE

The generation of O-2A^{adult} progenitor cells by O-2A^{perinatal} progenitor cells appears not to play a role in the maintenance of the O-2A^{adult} progenitor population in the optic nerves of adult animals, as indicated by the observation that the O-2A^{perinatal} progenitor population eventually disappears from the developing optic nerve (Wolswijk and Noble, 1989) (see Figure 9-2). Current evidence suggests that O-2A^{adult} progenitor cells express properties of stem cells, thereby allowing these cells to support their own replenishment as well as that of the oligodendrocyte population throughout life (for reviews on stem cells, see Hall and Watt, 1989; Potten and Loeffler, 1990). One stem cell-like property of O-2A^{adult} progenitor cells is the expression of long cell cycle times (Wolswijk and Noble, 1989; Wolswijk et al., 1991b; Wren et al., 1992). A further stem cell-like property of O-2A^{adult} progenitor cells is their apparent ability to undergo asymmetric division and dif-

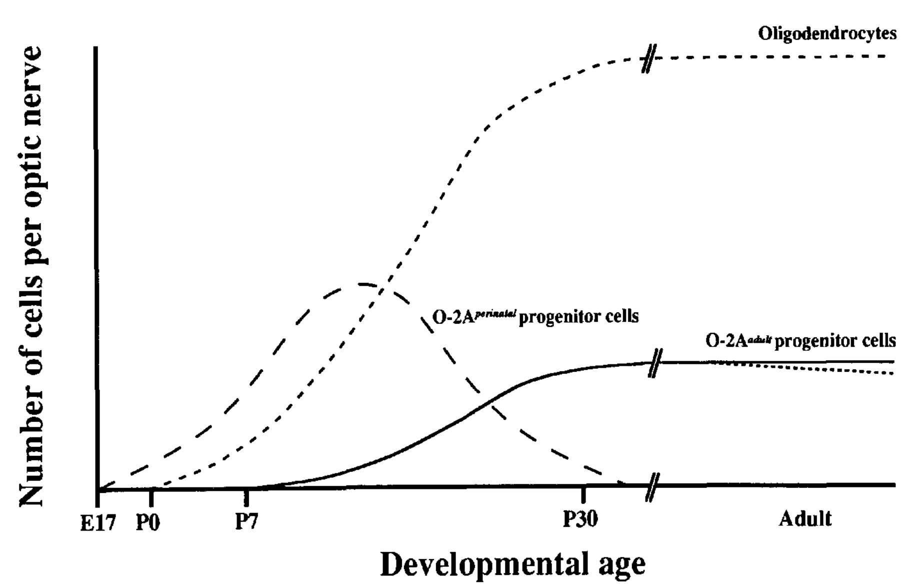


FIG. 9-2. Developmental transition in O-2A progenitor type during development of the rat optic nerve. Indirect evidence suggests that O-2A^{perinatal} progenitor cells migrate into the developing nerve at approximately embryonic day 17 (*E17*) (Small et al., 1987), while their differentiated progeny, oligodendrocytes, are first seen at around the day of birth (P0) (Miller et al., 1985). O-2A^{adult} progenitor cells appear in the nerve approximately 7 days after birth and this population of cells coexists with O-2A^{perinatal} progenitor cells until the *perinatal* progenitor population has disappeared from the nerve (Wolswijk and Noble, 1989; Wolswijk et al., 1990). The O-2A^{adult} progenitor population persists into adulthood, though there are suggestions that their number may gradually decline with age (Wolswijk and Noble, 1989; 1992; G. Wolswijk, unpublished observations). The relative cell numbers that are given are not indicative of the actual numbers of O-2A^{perinatal} progenitor cells, O-2A^{adult} progenitor cells and oligodendrocytes in the developing and adult optic nerve, but are meant to reflect the relative fluctuations in these populations as indicated by current data.

ferentiation. Indirect evidence for this has come from studies in which the composition of colonies generated by single O-2A^{adult} progenitor cells was analysed. These studies showed that oligodendrocytes tend to coexist in the same colony as dividing O-2A^{adult} progenitor cells (Wren et al., 1992). Asymmetric division and differentiation of O-2A^{adult} progenitor cells grown *in vitro* has also been demonstrated directly using time-lapse cinemicroscopy, as illustrated in Figure 9-3. This figure shows that 14 clonally-related O-2A^{adult} progenitor cells had different fates: some O-2A^{adult} progenitor cells in this particular family differentiated into oligodendrocytes, while others continued to divide at least four more times as O-2A^{adult} progenitor cells. Oligodendrocytes were generated also in branches of the family that contained dividing O-2A^{adult} progenitor cells and this occurred in an asymmetrical fashion.

In contrast to the stem cell-like O-2A^{adult} progenitor cells, O-2A^{perinatal} progenitor cells appear to be true progenitor cells, committed to differentiate within a limited time period, both *in vitro* and *in vivo*. Single O-2A^{perinatal} progenitor cells derived from P0 to P7 optic nerves and grown in microwells in Astro-CM or at low density on monolayers of cortical astrocytes normally divide ≤ 8 times before all cells in the colony differentiate clonally and synchronously into oligodendrocytes (Temple and Raff, 1986; Wren et al., 1992). A small number of col-

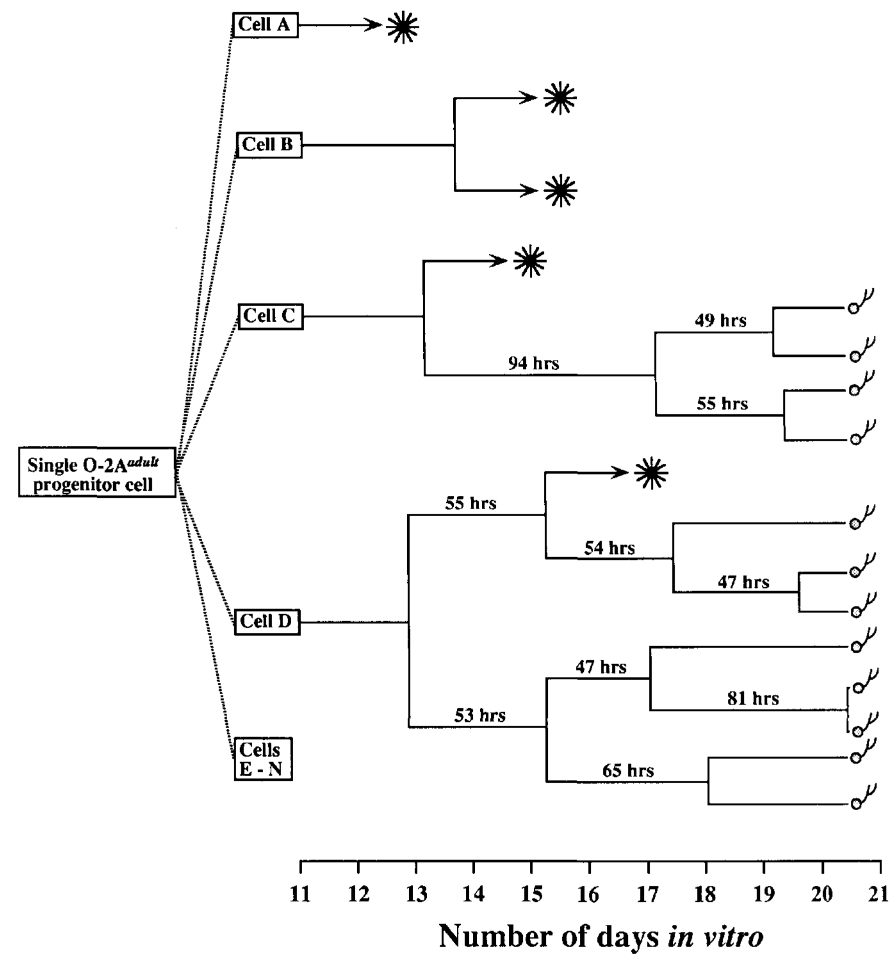


FIG. 9-3. Direct visualization of the asymmetric mode of division and differentiation of O-2A^{adult} progenitor cells grown on cortical astrocytes. Adult optic nerve cells were plated at low density on monolayers of cortical astrocytes (see Wolswijk and Noble, 1989). A colony generated by a single O-2A^{adult} progenitor cells was initially followed on a daily basis using an inverted microscope. When this colony was followed further using time-lapse cinemicroscopy, it was found that the 14 cells that were present in the O-2A^{adult} progenitor colony at the start of the filming period (cells *A–N*) had different fates (only the fate of cells *A–D* are shown). For example, *Cell A* adopted a multipolar oligodendrocyte-like morphology shortly after the start of the filming period, while *Cell B* divided once and both of its progeny cells acquired an oligodendrocyte-like morphology during the filming period. *Cell C* and *Cell D* generated many more progeny cells and some of these were still proliferating at the end of the filming period. Both cells also generated oligodendrocyte-like cells, and this occurred in an asymmetrical fashion. Thus, these results provide direct evidence that an O-2A^{adult} progenitor cell can generate oligodendrocytes and O-2A^{adult} progenitor cells in an asymmetrical manner. At the end of the filming period, this particular O-2A^{adult} progenitor colony contained 31 O-2A^{adult} progenitor cells and 15 oligodendrocyte-like cells. To illustrate the slow division rate of O-2A^{adult} progenitor cells grown in the presence of cortical astrocyte-derived factor(s) (Wolswijk and Noble, 1989), the cell cycle time of the various O-2A^{adult} progenitor cells in this family have been indicated.

onies generated by single O-2A^{perinatal} progenitor cells continue to expand following the appearance of oligodendrocytes (Temple and Raff, 1986; Wren et al., 1992), but such colonies may have generated O-2A^{adult} progenitor cells. Thus, O-2A^{adult} and O-2A^{perinatal} progenitor cells also differ in the manner these cells generate oligodendrocytes *in vitro:* most proliferating O-2A^{perinatal} progenitor cells differentiate into oligodendrocytes in a clonal fashion, while proliferating O-2A^{adult} progenitor cells predominantly generate oligodendrocytes in an asymmetrical manner (Table 9-1).

REPAIR PHENOTYPE OF O-2A^{adult} PROGENITOR CELLS

The presence of a population of oligodendrocyte progenitor cells in the adult CNS suggests that these cells may play an important role in the replenishment of the oligodendrocyte population following demyelinating damage, possibly in addition to mature oligodendrocytes (see later). This view is supported by two observations: First, cells with the antigenic characteristics of O-2A^{adult} progenitor cells grown *in vitro* have been shown to incorporate [^{3}H]thymidine and/or increase in number during the recovery process from demyelinating damage (Godfraind et al., 1989; Carrol et al., 1990). Second, the oligodendrocyte lineage cells that proliferate in response to experimentally-induced demyelination (Ludwin, 1979, 1984; Aranella and Herndon, 1984; Carrol et al., 1990; Rodriguez et al., 1991) and those that are present at the edges of demyelinating lesions in patients suffering from the most common human demyelinating disease multiple sclerosis (Raine et al., 1981) are ultrastructurally similar to O-2A^{adult} progenitor cells grown *in vitro* (Wolswijk et al., 1991a).

If the slowly dividing, stem cell-like O-2A^{adult} progenitor cells replenish the oligodendrocyte population following demyelinating damage, these cells may then also express another property of stem cells, that is, the ability to generate rapidly large numbers of progeny cells (Hall and Watt, 1989; Potten and Loeffler, 1990). Moreover, if O-2A^{adult} progenitor cells are involved in damage repair, substances involved in stimulating the division of O-2A^{adult} progenitor cells must be expressed or released following injury.

Several molecules present in lesion sites could trigger the increased proliferation of oligodendrocyte lineage cells in response to demyelinating damage, including cytokines secreted by reactive astrocytes, inflammatory cells or microglia, myelin breakdown products, and components present on demyelinated axons. However, due to the complexity of the *in vivo* situation, it is virtually impossible to determine which factors promote regeneration of the oligodendrocyte population, which factors hamper this, and which factors have no effect. Therefore, recent studies on O-2A^{adult} progenitor cells have been concerned with determining whether some of the factors in lesion sites are capable of inducing rapid division in O-2A^{adult} progenitor cells grown *in vitro*.

A factor that both appears to be expressed at increased levels following injury to the adult central nervous system (Finkelstein et al., 1988; Logan, 1988, 1990; Nieto-Sampedro et al., 1988; Eckenstein et al., 1991; Gómez-Pinilla et al., 1992; Logan et al., 1992) and is capable of increasing the rate of division of O-2A^{adult} progenitor cells grown *in vitro* is basic fibroblast growth factor (bFGF) (Wolswijk and Noble, 1992). Time-lapse cinemicroscopy studies have shown that O-2A^{adult} progenitor cells grown in bFGF have significantly shorter cell cycle times than those cultured in PDGF or in the presence of cortical astrocytes (Wolswijk and Noble, 1992). In addition, bFGF also almost completely prevents the differentiation of O-2A^{adult} progenitor cells into oligodendrocytes. The effects of bFGF on O-2A^{adult} progenitor cells appear to be direct, as such cells bind both radiolabeled bFGF and anti-FGF receptor antibodies (Wolswijk and Noble, 1992).

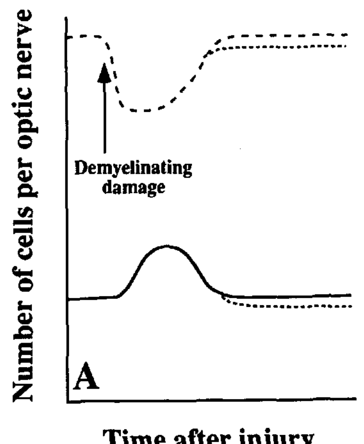
O-2A^{adult} progenitor cells exposed to bFGF can be induced to divide even more rapidly if they are also exposed to PDGF (Wolswijk and Noble, 1992). Moreover, many O-2A^{adult} progenitor cells grown in both PDGF and bFGF also express other characteristics of O-2A^{perinatal} progenitor cells, such as high rates of migration, bipolar morphology, and O4$^-$vimentin$^+$ antigenic phenotype (Wolswijk and Noble, 1992). Thus, cooperation between PDGF and bFGF converts many slowly dividing O-2A^{adult} progenitor cells to rapidly dividing cells with characteristics of O-2A^{perinatal} progenitor cells.

The effects of bFGF on the properties of O-2A^{adult} progenitor cells appear to be transient and bFGF does not induce rapid division and migration in all O-2A^{adult} progenitor cells (Wolswijk and Noble, 1992). However, as long as cultures are exposed to bFGF, O-2A^{adult} progenitor cells are prevented from differentiating into oligodendrocytes, even after 3 months of exposure (Wolswijk and Noble, 1992; G. Wolswijk, unpublished observations). The transient nature of the effect of bFGF on O-2A^{adult} progenitor cells could reflect intrinsic limitations on the growth of these cells, or it is possible that O-2A^{adult} progenitor cells need the presence of additional factor(s) to continue expressing an O-2A^{perinatal} progenitor-like phenotype.

If the pattern of cellular behavior expressed by O-2A^{adult} progenitor cells exposed to bFGF *in vitro* is duplicated *in vivo* following demyelinating damage (Figure 9-4), it would allow rapid increases in the number of O-2A^{adult} progenitor cells [as observed, for example, in lesions of animals recovering from experimentally-induced demyelination (Godfraind et al., 1989; Carrol et al., 1990)]. Subsequent differentiation of O-2A^{adult} progenitor cells into oligodendrocytes, followed by re-ensheathement of the denuded axons could then restore proper impulse conduction leading to recovery of function. Consistent with the hypothesis that bFGF may be involved in the increased generation of O-2A^{adult} progenitor cells *in vivo* are the findings that mechanically-induced injury to the adult central nervous system is associated with transient increases in the levels of bFGF, PDGF and their mRNAs (Finklestein et al., 1988; Logan, 1988, 1990; Nieto-Sampedro et al., 1988; Eckenstein et al., 1991; Gómez-Pinilla et al., 1992; Logan et al., 1992; Lotan and Schwarz, 1992). However, it is not yet known whether demyelinating damage is associated with increases in the levels of these two growth factors, either in experimental models of demyelinating diseases or in patients with multiple sclerosis.

The cellular and molecular mechanisms that underlie regenerative events in the oligodendrocyte lineage of the adult central nervous system suggest the following hypothesis (see Figures 9-4 and 9-5). PDGF is important in controlling the proliferation, migration, and differentiation of O-2A^{adult} progenitor cells in the healthy adult central nervous system. O-2A^{adult} progenitor cells in the normal adult brain either do not see bFGF or are exposed to only very low concentrations of bFGF. Following demyelinating damage, O-2A^{adult} progenitor cells in lesion sites are now exposed to much higher concentrations of PDGF and bFGF, resulting in the expression of a *repair* phenotype by many O-2A^{adult} progenitor cells. When the levels of bFGF (and PDGF) have gone down again, many O-2A^{adult} progenitor cells stop dividing and generate the oligodendrocytes that are needed to repair demyelinated lesions. Basic FGF in lesion sites may also induce division in oligodendrocytes that have survived demyelinating damage or in newly generated oligodendrocytes, as *in vitro* studies have suggested that bFGF is a mitogen for oligodendrocytes (see later).

The increased levels of PDGF seen following injury may have an additional function with regards to the generation of new oligodendrocytes in response to demyelinating damage. Studies by Barres



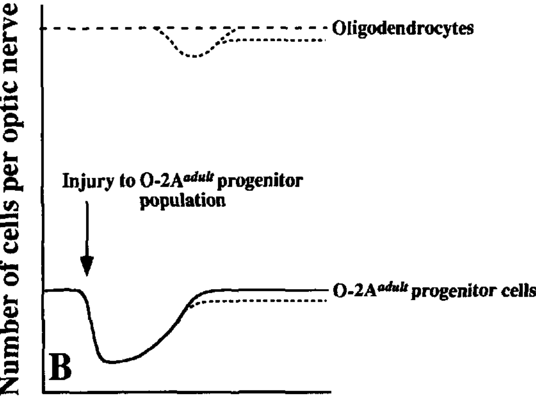


FIG. 9-4. Model for the regenerative events that may occur in the O-2A lineage of the adult central nervous system following damage to the oligodendrocyte population or O-2A^{adult} progenitor population. (A) Demyelinating damage to the adult central nervous system induces the expression of a rapidly dividing phenotype in O-2A^{adult} progenitor cells, resulting in a local increase in the number of O-2A^{adult} progenitor cells. When sufficient new O-2A^{adult} progenitor cells have been generated, these cells differentiate into oligodendrocytes, which in turn remyelinate the denuded axons. It theoretically is possible that demyelinating damage may result in a permanent reduction in the number of O-2A^{adult} progenitor cells (and oligodendrocytes). (B) There is evidence that also the O-2A^{adult} progenitor population is replenished when this population is damaged specifically. Studies by Van der Maazen and colleagues have indicated that x-ray irradiation of the adult optic nerve *in vivo* results in a dramatic decrease in the number of O-2A^{adult} progenitor cells that can be recovered from irradiated adult optic nerves shortly following irradiation (Van der Maazen et al., 1992). With time the number of O-2A^{adult} progenitor cells that can be isolated from irradiated optic nerves increases, but even low doses of x-ray irradiation cause a permanent reduction in the number of O-2A^{adult} progenitor cells (Van der Maazen et al., 1992). This in turn could then theoretically also cause a temporary or permanent reduction in the number of oligodendrocytes. Consistent with this hypothesis is the observation that demyelination can be one of the side effects of x-ray irradiation *in vivo* (Van der Kogel, 1980).

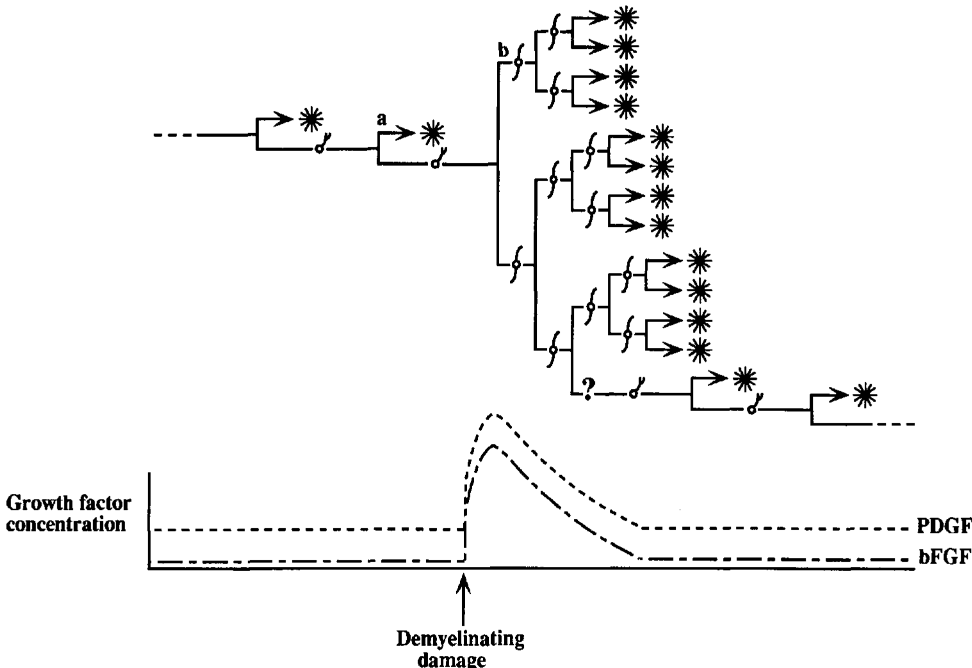


FIG. 9-5. A model of the generation of large numbers of oligodendrocytes by O-2A^{adult} progenitor cells in response to demyelinating damage. In the healthy adult central nervous system, O-2A^{adult} progenitor cells are exposed to PDGF (and possibly low levels of bFGF) and generate oligodendrocytes in an asymmetrical fashion and at a slow rate [To simplify the model, each O-2A^{adult} progenitor cell was assumed to generate only one oligodendrocyte and one further O-2A^{adult} progenitor cell at each division]. Following injury to the central nervous system, the levels of PDGF and bFGF are increased transiently. As a result, O-2A^{adult} progenitor cells are exposed to much higher concentrations of bFGF and PDGF and this elicits the expression of a *repair* phenotype in many O-2A^{adult} progenitor cells: instead of differentiating, O-2A^{adult} progenitor cells continue to divide and do so with much shorter cell cycles than when these cells are exposed only to PDGF. When the levels of PDGF and bFGF have gone down again, the O-2A^{adult} progenitor cells that kept on dividing in response to PbF differentiate and generate the oligodendrocytes that are needed to repair demyelinated lesions. For example, *Cell b*, in this model, generates four oligodendrocytes instead of the one oligodendrocyte generated by *Cell a*. The increased levels of PDGF may also be important in increasing the survival of newly generated oligodendrocytes (see Barres et al. 1992). It is not yet known whether all the members of an O-2A^{adult} progenitor family following transient exposure to PbF differentiate into oligodendrocytes or whether some cells remain as O-2A^{adult} progenitor cells.

and colleagues have suggested that about 50% of newly formed oligodendrocytes in the optic nerves of developing animals die due to limiting amounts of PDGF (Barres et al., 1992). If 50% of the newly generated oligodendrocytes in the adult central nervous system also normally die, increased survival of oligodendrocytes as a result of local increases in the concentrations of PDGF in itself could significantly increase the numbers of oligodendrocytes available to repair demyelinating lesions.

THE SIGNIFICANCE OF THE DEVELOPMENTAL TRANSITION IN O-2A PROGENITOR TYPE

The properties expressed by O-2A^{perinatal} progenitor cells *in vitro* are consistent with the expected physiological requirements of the developing central nervous system. For example, the rapid rate of division of O-2A^{perinatal} progenitor cells grown *in vitro* is consistent with evidence indicating that O-2A lineage cells are generated rapidly during development of the rat optic nerve (Skoff et al., 1976a, 1976b; Miller et al., 1985; Barres et al., 1992). Furthermore, the highly motile nature of O-2A^{perinatal} progenitor cells is in agreement with suggestions that these cells migrate from germinal zones into several regions of the developing central nervous system such as optic nerve (Small et al., 1987), cerebellum (Wolswijk, 1995), and spinal cord (Warf et al., 1991). Finally, the observation that O-2A^{perinatal} progenitor families either differentiate clonally into oligodendrocytes or generate O-2A^{adult} progenitor cells is consistent with the finding that the O-2A^{perinatal} progenitor population disappears from the developing optic nerve (see Figure 9-2).

The *in vitro* properties of O-2A^{adult} progenitor cells are also consistent with a number of *in vivo* obser-

vations. First, there appears to be only a modest production of oligodendrocytes in the adult brain (McCarthy and Leblond, 1988), which is consistent with the slow rate of division and differentiation of O-2A^{adult} progenitor cells. Second, the observation that demyelinating damage appears to be associated with increased division in the oligodendrocyte lineage is compatible with the ability of O-2A^{adult} progenitor cells to express a rapidly dividing phenotype. Moreover, it is reasonable to propose that the normal mature brain has a reduced requirement for precursor migration, which is in agreement with the slow migratory behavior of O-2A^{adult} progenitor cells *in vitro*. However, when O-2A^{adult} progenitor cells are induced to undergo rapid division, these cells acquire the ability to migrate rapidly and this increased migratory capacity may be relevant to repair of demyelinated lesions.

PROLIFERATION OF OLIGODENDROCYTES AND RECOVERY FROM DEMYELINATING DAMAGE

In addition to the generation of new oligodendrocytes by O-2A^{adult} progenitor cells in response to demyelinating damage, proliferation of oligodendrocytes may be important in the recovery process. Uptake of [^{3}H]thymidine by oligodendrocytes has been observed *in vivo* after experimentally-induced demyelination (Aranella and Herndon, 1984) and after mechanical trauma to the adult brain (Ludwin, 1984). In addition, Ludwin and Bakker (1988) found that even myelinating oligodendrocytes incorporated radiolabeled thymidine after wounding of the cerebral cortex of adult mice. However, only a comparatively small proportion of oligodendrocytes were radiolabeled in these studies, suggesting that their contribution to the generation of new oligodendrocytes may be relatively small. It is not known what factor(s) induce division in oligodendrocytes *in vivo*.

The ability of oligodendrocytes to divide has been demonstrated directly in tissue culture experiments. For example, oligodendrocytes incorporate [^{3}H]thymidine in response to bFGF (Eccleston and Silberberg, 1985; Saneto and DeVellis, 1985; Bögler et al., 1990; Wolswijk and Noble, 1992), when cocultured with dorsal root ganglion cells (Wood and Bunge, 1986, 1991), when exposed to axollemal-enriched fractions (Chen and DeVries, 1989) and when grown on an extracellular matrix (Ovadia et al., 1984). However, the vast majority of the oligodendrocytes that are induced to divide by exposure to bFGF and those that proliferate when cocultured with dorsal root ganglion neurons do not express myelin basic protein (MBP) (Wood and Bunge, 1986, 1991; D. Wren and M. Noble, unpublished observations), a marker for mature, myelinating oligodendrocytes. These observations suggest that only a population of immature oligodendrocytes is proliferatively active, at least *in vitro*.

FACTORS LIMITING MYELIN REPAIR

There are several factors that may hamper regeneration in the oligodendrocyte lineage, some of which may be relevant to the ultimate failure of myelin repair in patients suffering from multiple sclerosis (McDonald and Silberberg, 1986). While there is some evidence for myelin repair at the edges of multiple sclerosis lesions (Prineas and Connel, 1979; Raine et al., 1981; Prineas et al., 1989), the centers of old lesions of "plaques" are often virtually completely devoid of oligodendrocytes, and bare axons are surrounded instead by astrocytic processes. Very little is known about why remyelination is generally unsuccessful in multiple sclerosis patients or about the cause of the initial destruction of oligodendrocytes. Although great caution should be taken with extrapolating tissue culture data obtained with rat cells to humans, it is intriguing that *in vitro* studies have revealed striking consistencies between the properties of O-2A^{adult} progenitor cells and certain features of multiple sclerosis.

Exposure of O-2A^{adult} progenitor cells (and oligodendrocytes) to a factor that specifically eliminates these cells *in vivo* would theoretically contribute to the formation of lesions that remain chronically demyelinated (see Figure 9-4), such as those found in multiple sclerosis patients. An example of such a possibility has come from *in vitro* studies that have indicated that O-2A^{adult} progenitor cells share with oligodendrocytes the ability to bind and activate complement in the absence of antibody, resulting in their destruction (Wren and Noble, 1989). This sensitivity to complement appears to be a specific property of oligodendrocytes and O-2A^{adult} progenitor cells, as several other cell types, including O-2A^{perinatal} progenitor cells, do not express this property (Scolding et al., 1989; Wren and Noble, 1989) (see Table 9-1). While it is not known whether complement-mediated destruction of O-2A^{adult} progenitor cells and oligodendrocytes plays a role in the formation of chronically demyelinated lesions, complement does appear to be activated in areas of myelin breakdown (Compston et al., 1991).

It appears that both the number of O-2A^{adult} progenitor cells that can be obtained from one pair of adult optic nerves and the number of cells O-2A^{adult}

progenitor cells give rise to *in vitro* when stimulated to divide by cortical astrocytes decreases with age of the donor animal (Wolswijk and Noble, 1989, 1992; Van der Maazen et al., 1992; G. Wolswijk, unpublished observations). These data suggest that with increasing age fewer O-2A^{adult} progenitor cells may be available to generate the oligodendrocytes that are needed to repair demyelinating damage, thereby reducing the ability of the adult central nervous system to recover from demyelinating damage. This is complemented by two *in vivo* observations. First, the potential for recovery from optic neuritis, a human demyelinating disease of the optic nerve, appears to decrease with age (McDonald, 1983; Kriss et al., 1988). Second, ethidium bromide-induced demyelinated lesions in spinal cord white matter of adult rats are repaired less successfully in rats that are over 12 months old as compared to 5-month-old rats (Gilson and Blakemore, 1993).

THE *IN VIVO* IDENTIFICATION OF O-2A^{adult} PROGENITOR CELLS

To study the role of O-2A^{adult} progenitor cells in the repair of demyelinated lesions *in vivo*, the identification of such cells in sections of adult central nervous system tissue is essential. Several potential markers for O-2A^{adult} progenitor cells *in vivo* have been identified. For example, Pringle and colleagues have suggested that O-2A^{adult} progenitor cells in sections of adult optic nerve can be identified on the basis of their expression of the PDGF α-receptor (Pringle et al., 1992), while Fulton and colleagues have suggested that glutamate receptor-linked cation channels of the quisqualate-kainate type may be expressed by only O-2A^{adult} progenitor cells in the adult central nervous system (Fulton et al., 1992). Another study has suggested that O-2A^{adult} progenitor cells in the adult cerebellum express the NG2 chondroitin-sulfate proteoglycan (Levine et al., 1993; Levine 1994).

A recent study has examined whether antibodies to the ganglioside G_{D3} can be used to identify O-2A^{adult} progenitor cells in sections of adult central nervous system tissue, as studies on the developing central nervous system had suggested that O-2A^{perinatal} progenitor cells are G_{D3}^+ *in vivo* (e.g., Reynolds and Wilkin, 1988). Although O-2A^{adult} progenitor cells express G_{D3} in tissue culture, all G_{D3}^+ cells in sections of adult optic nerve are ramified microglia (Wolswijk, 1994). Moreover, a re-examination of the identity of G_{D3}^- expressing cells in the developing cerebellum has revealed that all strongly G_{D3}^+ cells are either amoeboid microglia or ramified microglia and not O-2A^{perinatal} progenitor cells (Wolswijk, 1995), as suggested previously (e.g., Reynolds and Wilkin, 1988). Thus, these results suggest that the identification of a cell as an O-2A^{adult} progenitor cell (or any other cell type) on the basis of the expression of a single marker may not be entirely reliable. Therefore, it is of paramount importance to re-examine the studies that have identified markers for O-2A^{adult} progenitor to verify that the cells that express these markers *in vivo* are indeed O-2A^{adult} progenitor cells.

CONCLUSIONS

A number of conclusions can be drawn from the *in vitro* studies on O-2A^{perinatal} and O-2A^{adult} progenitor cells. *First*, the developing and adult central nervous system each contain a population of O-2A progenitor cells with properties that appear to be consistent with the expected physiological requirements of their respective environments. *Second*, O-2A^{perinatal} and O-2A^{adult} progenitor cells belong to the same glial lineage. *Third*, O-2A^{perinatal} progenitor cells are true progenitor cells, while O-2A^{adult} progenitor cells express properties of stem cells. *Fourth*, cooperation between PDGF and bFGF, two factors that appear to be upregulated and/or released following central nervous system injury, induces a *repair* phenotype in O-2A^{adult} progenitor cells. *Fifth*, studies on O-2A^{adult} progenitor cells have provided clues as to why remyelination may be less effective in adults with multiple sclerosis than in children with similar lesions. *Sixth* and finally, as there also appear to be differences between *perinatal* and *adult* precursor populations in other lineages of multicellular organisms (e.g., Metcalf and Moore, 1971; Wood et al. 1985; Anderson, 1989; Stockdale, 1992), the studies on O-2A^{perinatal} and O-2A^{adult} progenitor cells could serve as a model to provide useful strategies for the analysis of developmental transitions in precursor populations in such lineages.

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10 Development of microglia

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Microglia are a significant component of the fabric of the central nervous system (CNS). They comprise 5 to 12% of the cells of the central nervous system and are arranged strategically and in an orderly topographical manner (Perry et al., 1985; Lawson et al., 1990). Recent investigations point to a pivotal role for microglia, not only in the defense mechanism of the CNS, but also in its steady-state condition (Graeber and Streit, 1990; Merrill, 1991, 1992; Fedoroff et al., 1993). Despite the important role of microglia in the neuron-glia functional loop, their origin, lineage, and mode of differentiation are still cloaked in mystery and beset with misconceptions. A longstanding controversy centers around the origin of microglia: are they descendants of hemopoietic myeloid stem cells or, like other glia, do they form from neuroectoderm?

Early work on the subject is well summarized in a number of reviews: Rio Hortega (1932); Glees (1955); Kitamura (1973); Oehmichen (1978); Boya et al. (1979); Baldwin (1980); Fujita (1980); Fujita et al. (1981); Ling (1981); Polak et al. (1982); Jordan and Thomas (1988); Streit et al. (1988); Perry and Gordon (1991); Peters et al. (1991); Thomas (1992); Ling and Wong (1993); Theele and Streit (1993). The earlier studies were predominantly based on morphological observations using selective silver carbonate impregnation, electron microscopy, [^{3}H]TdR incorporation into the DNA of cells, and by "marking" phagocytic cells with colloidal carbon. More recent studies utilize chimeric animals in which hemopoietic cells carry an identifiable protein or DNA genetic marker, immunocytochemistry, and developmental and functional analysis in tissue culture.

GENERAL CONSIDERATIONS

A popular view is that microglia form from hemopoietic monocytes; therefore, this chapter first reviews the development of the hemopoietic system in general, the development of monocytes/macrophages, and more recent work relevant to the understanding of the development and cell lineage of microglia. These topics are closely interrelated, and, to keep the origin of microglia in focus while discussing them, three theoretical possibilities for microglia formation in the CNS are described and their feasibility considered.

Colonization of the Central Nervous System by Hemopoietic Stem Cells During Ontogeny

In this possible situation, the colonizing cells would be hemopoietic stem cells that can self-replicate and be able to provide differentiating cells throughout the life of the animal to maintain a constant number of microglia in the CNS. Such stem cells would have to enter the CNS during the early stages of embryonic development and either be dispersed throughout the CNS or be located in discrete "germinal centers." Hemopoietic centers that produce the various blood cells are not known to exist in the CNS, and therefore it would have to be assumed that the parenchyma of the CNS is compatible with hemopoietic stem cell survival but inhibits the stem cells from forming hemopoietic cells other than in the monoblast-monocyte lineage. If the colonizing hemopoietic cells were already restricted to the monoblast-monocyte lineage before they colonized the CNS, such cells would be expected to have a limited degree of proliferation and would be considerably modified phenotypically from their counterparts in the bone marrow.

The self-replicating hemopoietic stem cells in the CNS would have to produce a number of transitional forms ranging from monoblasts to monocytes and then from monocytes to microglia. As microglia populate the entire CNS in an orderly manner, they would have to migrate extensively to find their proper location. There would also have to be a regulatory mechanism for the turnover rate of microglia under steady-state conditions to maintain constant numbers of microglia in the CNS and under pathological conditions to rapidly increase the numbers of microglia.

Infiltration of the Central Nervous System by Hemopoietic Cells Throughout the Life of the Animal

In this scenario the self-replicating stem cells would be located outside the CNS, most likely in the bone marrow, and their progeny (monocytes) would infiltrate the CNS throughout the life of the animal. The infiltrating cells either would have limited ability to divide and produce a number of transitional forms in the CNS that would eventually form microglia or would transform directly into microglia, as monocytes transform into macrophages in the body tissues. Under this possibility, as in the previous one, the immature cells would have to be migratory. The regulating mechanism for microglia turnover would have to be responsible for the normal steady-state condition in the hemopoietic tissue. From this it follows that deviation from the normal steady state condition in the hemopoietic tissue would reflect the turnover of microglia within the CNS.

Development of Microglia from Neuroectoderm

This possibility would assume that microglia originate from a common glia stem cell, the glioblast, in the ventricular and later, in the subventricular zone of the developing neural tube. The microglia lineage would be a branch of the glia lineage and precursor cells responsible for the turnover rate of astroglia and oligodendroglia throughout the life of the animal might also be responsible for the turnover rate of microglia.

These are the three most obvious possibilities for the origin of microglia. We now examine pertinent available information to reach some conclusions as to which possibilities are tenable.

DEVELOPMENT OF THE HEMOPOIETIC SYSTEM

In mammals, the ancestral hemopoietic stem cells form extraembryonically in the wall of the yolk sac (Metcalf and Moore, 1971). There are indications, however, that the ancestral hemopoietic stem cells may have multiple sites of origin. It has been well demonstrated that in the chick, ancestral hemopoietic stem cells form independently, intraembryonically, in the dorsal wall of the thoracic aorta as well as in the yolk sac. Similar observations have been made in mice (Smith and Glomski, 1982). From both sites the stem cells colonize other hemopoietic centers (Dieterlen-Lièvre, 1987; Cormier and Dieterlen-Lièvre, 1988; Dieterlen-Lièvre et al., 1988). Ogawa et al. (1988) suggested that stem cells producing B lymphocytes arise *de novo* within the embryo. B-lymphocytes and myeloid cells arise from the same primitive stem cell; it therefore follows that some pluripotent stem cells must originate intraembryonically. Further support for this notion was recently provided by Cumano et al. (1992). They unequivocally demonstrated that in the livers of mice at day 12 of gestation, bipotential cells are present that give rise to B lymphocytes and macrophages. This observation is of great significance because it indicates that macrophages can form from several lineages, not only via the monoblast-monocyte lineage.

In mice, the first signs of erythropoiesis are seen at Theiler stage 11 (day 7 of gestation) and in man on days 21 to 28 (Metcalf and Moore, 1971; Theiler, 1972). Although the stem cells in the yolk sac produce only nucleated erythrocytes, they have the potential to form cells of all hemopoietic cell lineages. This has been verified by analyzing the stem cells in tissue cultures and/or irradiated animals (Moore and Metcalf, 1970; Metcalf and Moore, 1971; Perah and Feldman, 1977; Migliaccio et al., 1986). When the cardiac tubes begin to contract and the blood islands of the yolk sac become connected to the intraembryonic blood vascular network, the stem cells from the yolk sac enter the circulation on day 11 of gestation in mice and at 5 weeks postconception in humans (Metcalf and Moore, 1971; Migliaccio et al., 1986). They colonize the mesodermal hepatic rudiment where enucleated erythrocytes are beginning to be produced. At first only enucleated erythropoiesis takes place in the hepatic rudiment but later, macrophages and granulomonocytic elements appear. It has been suggested that at this stage some macrophages may be forming from mesenchymal cells and/or granulomonocytic stem cells, which preferentially differentiate in the monocyte pathway (Migliaccio et al., 1986).

The mesenchymal spleen rudiment in mice appears on day 13 of gestation and hemopoietic stem cells from the liver colonize the spleen and begin active erythropoiesis in the spleen at day 15. In humans the spleen is populated by stem cells at 19 weeks (Hann et al., 1983). By day 17, in mice, granulopoiesis predominates. The spleen is a unique hemopoietic organ in which every hemopoietic cell type is produced. It has been estimated that on day 4 postnatal, mouse spleen contains about 50% of the total body population of hemopoietic stem cells (Metcalf and Moore, 1971).

The bone shafts in mice are cartilaginous at day 15 of gestation. On day 17, marrow cavities appear and by day 17–18 they are colonized by circulating stem cells from the liver and spleen. Erythropoiesis, however, does not begin before birth. In human embryos, hemopoiesis begins around 15–16 weeks (Hann et al., 1983) or according to Metcalf and

Moore (1971), at the 20th week of gestation. Once the bone marrow has become colonized by hemopoietic stem cells, further development proceeds by the expansion of the stem cell population rather than by recruitment of stem cells from liver and spleen.

DEVELOPMENT OF MONOCYTOPOIESIS

Several types of multipotential, self-replicating, hemopoietic stem cells generate a progeny of eight major lineages of hemopoietic cells that form erythrocytes, megakarocytes, monocytes, granulocytes, basophils, eosinophils, T lymphocytes, and B lymphocytes. The stem cell progeny progress along various pathways of differentiation in a rather heterogeneous fashion but always toward more and more committed cells with restricted developmental potential and less and less proliferative capacity. The monoblast-monocyte-macrophage lineage begins with myeloid-lymphoid stem cells, then pluripotential myeloid stem cells, followed by granulocyte-macrophage colony-forming cells, then macrophage colony-forming cells which differentiate into monoblasts, promonocytes, and finally monocytes (Novak et al., 1989). The number of cell generations between the ancestral stem cell and the generation of the final nondividing monocyte is not known. Monocytes remain in the bone marrow for probably less than 24 hours and then enter randomly into the blood circulation. There they marginate by attaching to the endothelium and then enter various body tissues, at different rates (van Furth, 1988). The half-time of monocytes in the blood circulation has been calculated as 17.4 hours (van Furth et al., 1973). Table 10-1 shows the percentage of monocytes leaving the circulation in different tissues and the rate of influx.

Formation of Macrophages During Fetal Development

Specific cell markers are available to identify monocytes-macrophages. The marker most commonly used is the F4/80 antibody to an unknown antigen (Austyn and Gordon, 1981), although recently it has been shown that eosinophils also bind antibody to F4/80 (McGarry and Stewart, 1991). Other markers include the Fc receptor for immunoglobulins, the CR3 receptor for complement (Unkeless and Wright, 1988), and α-D-galactose residues, which are recognized by *Griffonia simplicifolia* isolectin-B_4 (GSA I-B_4) (Streit et al., 1985; Streit, 1990). Thus monocyte-macrophage formation during embryonic development is defined in terms of these markers, mainly the expression of F4/80 antigen (Perry et al., 1985; Lawson et al., 1990; Gordon et al., 1992). Monocyte-macrophage development closely follows the development of hemopoiesis; however, it is curious that some F4/80-positive cells can be found in hemopoietic sites such as liver, spleen, and bone marrow, before hemopoiesis is seen (Morris et al., 1991). In mice, the F4/80 positive cells are clearly seen in the yolk sac between days 9 and 10 of gestation (Gordon et al., 1992), in liver at day 10–11, spleen at day 12, and bone marrow at day 16. In liver, spleen, and bone marrow there is a definite intimate association between monocytes-macrophages and erythroblasts (Morris et al., 1991). The development of macrophages is not, however, limited to the hemopoietic sites. They are found around the developing neuroectoderm and mesenchyme of lungs on day 12, thymus on day 14, and gut on day 15 (Morris et al., 1991). Macrophages are phagocytic but, contrary to the state in adult animals, proliferate profusely, and are found outside the blood vessels between mesenchymal cells. As the organs develop, macrophages become resident cells of the connective tissue of the organs (Morris et al., 1991).

Formation of Macrophages in Adult Tissues

Macrophages originate in adult body tissues and organ cavities from the monocytes that enter from the bone marrow. In the tissues, the monocytes increase in size and become ameboid. Their Golgi apparatuses enlarge and become the site of formation of

TABLE 10-1. *Kinetic Parameters of Macrophages at Various Sites*

Site	Monocytes leaving the circulation (%)	Rate of monocyte influx[a] ($\times 10^3$/h)	Rate of local production[a] ($\times 10^3$/h)	Mean turnover time of macrophages (days)
Liver	71.8	93.3 (92%)	7.7 (8%)	3.8
Spleen	24.7	15.2 (55%)	12.2 (45%)	6.0
Lung	14.7	9.1 (67%)	4.4 (33%)	6.0
Peritoneal cavity	6.7	4.2 (61%)	2.7 (39%)	14.9

From Van Furth (1988), with permission.

[a]The percentages in parentheses give the relative contribution made by influx and local production to the composition of the respective macrophage populations.

lysozyme and endocytotic activity increases. The transitional forms between monocytes and macrophages are difficult to define and all are based primarily on morphological characteristics. Although the majority of macrophages form from monocytes, there is evidence that certain numbers of macrophages form locally (Table 10-1), probably by division of preexisting macrophages or from other progenitor cells (Cormier and Dieterlen-Liévre, 1988; Ogawa et al., 1988; Cumano et al., 1992). The fate of macrophages is not known. However, there is no steady accumulation of macrophages in any tissues or organ cavities in the normal steady-state condition. There is some evidence that macrophages can translocate to other tissues or to regional lymph nodes where they die (van Furth et al., 1980). It is important to note that the estimated turnover time of body macrophages varies according to the tissue they are in and ranges from 3.8 days in the liver to 14.9 days in peritoneal cavity (van Furth, 1988) (Table 10-1).

The complex path of macrophage development and the various cell compartments evolved are in dynamic equilibrium. Hemopoiesis leading to the formation of mature blood cells, including macrophages, is regulated by a number of mechanisms. One depends on stromal cells (mesenchymal cell derivatives), which interact with hemopoietic cells at short range through trophic factors embedded in the stromal cell membranes or through soluble factors that signal the proximal cells (Dexter, 1982; Quesenberry et al., 1989). In addition, there are at least 16 distinct factors, (cytokines) produced by a variety of cells in hemopoietic sites as well as in other tissues. These trophic factors act on hemopoiesis by paracrine, autocrine, intracrine, juxtacrine, or signaling in synergistic, additive or inhibitory fashion. The interested reader is referred to the following reviews on this subject: Metcalf (1988, 1991); Orlic (1989); and Hamilton (1993).

ORIGIN OF THE CONCEPT OF THE MONONUCLEAR PHAGOCYTE SYSTEM

The term "macrophage" was coined by Metchnikoff (1892) to denote cells capable of ingesting large particles, including whole cells such as erythrocytes, spermatozoa, and the like, and to contrast them with polynuclear lymphocytes, which he termed "microphages" because they took up smaller particles such as bacteria. He recognized that macrophages in spleen, lymph nodes, and bone marrow have similar functions and that they are ameboid and in case of injury, surround the injured area or foreign body. He thought that such cells might serve in the defense of the organism against infections because they not only engulf but also rapidly kill and digest most invading microorganisms; he therefore introduced the term "macrophage system."

Aschoff (1924) modified and expanded the concept of a macrophage system. He grouped all cells that can take up vital dyes into the "reticuloendothelial system." He considered the uptake of vital dyes to be a test of phagocytosis and grouped cells in order according to the degree of dye uptake. He excluded endothelial cells and fibroblasts from the system because they take up little of the dyes. He included reticular cells of spleen and lymph nodes, reticuloendothelial cells of lymph and blood sinuses, including Kupffer cells, connective tissue histocytes, splenocytes, and monocytes. He pointed out that inclusion of cells in the reticuloendothelial system does not imply that they are identical; they may differ in their morphology, arrangement, and rate of uptake of foreign materials.

The practical significance of Aschoff's system and its heterogeneity was discussed in Leiden, Netherlands, during a conference on mononuclear phagocytes in 1949. It was felt that new knowledge of macrophage morphology, function, and kinetics makes it possible to group highly phagocytic mononuclear cells and their precursors into one system, the *mononuclear phagocyte system* (MPS) (van Furth et al., 1972).

A variety of experimental procedures have been used on normal animals and animals with acute or chronic inflammation, in lethally irradiated animals with or without bone marrow shielding, in radiation chimeras and parabiotic animals, using radioactive isotopes, chromosomes, or tissue antigens as stable markers to trace the cells. According to these studies, mononuclear phagocytes originate from precursor cells in the bone marrow, which are then transported by the peripheral blood as monocytes and eventually become tissue macrophages (van Furth et al., 1972, 1975). As a result the concept was developed that the mononuclear phagotype system is composed of cells related by similarities in morphology, function, and origin (van Furth et al., 1972, 1975).

The mononuclear phagocyte system includes monocytes and their precursor cells in the bone marrow, macrophages of the connective tissue, histiocytes, Kupffer cells of the liver, lung alveolar macrophages, free and fixed macrophages of lymph nodes, bone marrow macrophages, pleural and peritoneal cavity macrophages, osteoclasts, and microglia of the nervous system (van Furth, 1988). Microglia were included in the system with some reluctance. They were added because during the de-

velopment of the CNS, microglia cells are found only after the blood vessels have grown in (Andersen and Matthiessen, 1966), and in pathological processes, the accumulated mononuclear phagocytes supposedly including microglia originate from circulating monocytes (Konigsmark and Sidman, 1963; Kosunen et al., 1963; Waksman, 1965; Huntington and Terry, 1966; reviewed by van Furth, 1970; van Furth et al., 1972).

Indisputably, monocytes can infiltrate the CNS and have the potential to transform into macrophagelike cells (Meyermann et al., 1986; Wekerle et al., 1986; Hickey and Kimura, 1988; Hickey, 1991; Hickey et al., 1991; Hickey et al., 1992) and give rise to perivascular macrophages (Hickey and Kimura, 1988), but there is no convincing evidence that microglia in the parenchyma of the CNS form from monocytes (Choi, 1981; Matsumoto et al., 1985; Matsumoto and Ikuta, 1985; Schelper and Adrian, 1986; De Groot et al., 1992).

Despite efforts (van Furth et al., 1972, 1975) to unify the macrophages into a system of cells with common origin and function, the mononuclear phagocyte system represents a very heterogeneous population of cells (Gordon and Hirsch, 1982; Dijkstra et al., 1985; Nibbering et al., 1987; Perry and Gordon, 1987; van Furth, 1988; Keller et al., 1989; Oliver, 1990). The morphology of its cells varies greatly, depending on the tissue in which the cells are located and on their functional state. Even within the same tissue in normal steady-state conditions, the morphology of macrophages may differ. The antigenic characteristics vary in degree of expression and the frequency of cells expressing the antigen. Certain antigens may be absent in some macrophages and present in others (Walker, 1976; van Furth, 1988). Therefore, macrophages of each tissue must be characterized carefully; observations made with macrophages in one type of tissue do not necessarily apply to macrophages of another tissue. In tissue cultures, the morphology of macrophages can vary depending on the serum in the medium, concentration of bacterial wall lipopolysaccharide (endotoxin), and cytokines (e.g., CSF-1) (Graeber et al., 1989; Hao et al., 1990; Abd-El-Basset and Fedoroff, 1994).

There are, however, basic requirements for a cell to belong to the mononuclear phagocyte system (van Furth, 1980). These are as follows:

1. Morphological characteristics, including positive immunoreactivity with mononuclear phagocyte-specific antibodies, for example, F4/80, and the presence in the cytoplasm of such enzymes as nonspecific esterase, lysozyme, and peroxidase, which are demonstrable by histochemical and immunocytochemical methods.
2. Presence of Fc receptor.
3. Presence of CR3 receptor for complement.
4. Capability of Fc and CR3 receptors to mediate immunophagocytosis, and phagocytosis of opsinized bacteria.
5. Avid pinocytosis.

As a rule, not all of the mononuclear phagocytes are positive for all these criteria, but it is generally accepted that a cell must satisfy at least three of them before it can be considered to belong to the mononuclear phagocyte system (van Furth, 1988).

In order to make the point that cells that have common features and/or function do not necessarily have common ancestry, I would like to consider the dendritic cells. There is general hesitancy to include dendritic cells as full members of the mononuclear phagocyte system; instead, they are usually listed as belonging to the system—with a question mark (van Furth, 1988). The dendritic cells are present in most tissues of the body (see review by Fossum, 1989), although they are best known to occur in the lymphoid tissues and epidermis, where they are called Langerhans cells. Their common characteristics are that they express major histocompatibility complex (MHC) class II antigens, are antigen-presenting cells, are weakly phagocytic, and have veiled or dendritic morphology with a large amount of pale-staining cytoplasm. The Langerhans cells express Fc and CR3 receptors, and are F4/80-positive (Fossum, 1989; van Furth, 1988). Dendritic cells in human embryos have been found at 4 to 6 weeks of gestation together with monocytes and macrophages in the yolk sac (Janossy et al., 1986). They are disseminated from the yolk sac through all body tissues except cornea and brain (Hart and Fabre, 1981; Hofman et al., 1984). Dendritic cells are not found in embryonic or normal adult brains, but they do enter the brain from the blood circulation during allograft rejection (Lawrence et al., 1990).

During adult life dendritic cell precursor cells occur in the bone marrow where they proliferate. Dendritic cells are usually short lived in the tissues, although some may survive for several months (Fossum, 1989). They are renewed by cells from the bone marrow, although there is some evidence that Langerhans cells, especially, can replicate in tissues (Hashimoto and Tarnowski, 1968; Miyauchi and Hashimoto, 1987; review by Fossum, 1989). It has been estimated that 3 to 4% of Langerhans cells in the epidermis are in a cell cycle (Czernielewski et al., 1985). Thus, Langerhans cells are replenished from the bone marrow as well as locally in the epidermis.

During embryonic development dendritic cells are found in the same tissues, side by side with macrophages. This observation has been used to support the argument that dendritic cells belong to a separate cell lineage because it is not likely that the same environment would produce two phenotypically different cells stemming from the same progenitor cell (Fossum, 1989).

It is assumed that the development of hemopoietic cells is ordered in a hierarchical sequence, as mentioned previously in this chapter, starting with myeloid-lymphoid stem cells, pluripotential myeloid stem cells, granulocyte-monocyte colony forming cells (GM-CFCs), which give rise to macrophage-colony forming cells (M-CFCs) and granulocyte-colony forming cells (G-CFCs) (Novak et al., 1989). According to the mononuclear phagocyte system concept, the macrophages develop as terminally differentiated from M-CFCs, through a number of intermediate stages (van Furth, 1989). *In vitro* studies have indicated that the differentiation and function of M-CFCs is regulated by colony stimulating factor 1 (CSF-1) and the differentiation and function of G-CFCs by G-CSF. The differentiation of dendritic cells *in vitro,* however, is regulated by GM-CSF secreted by keratinocytes. CSF-1 and G-CSF have no effect on the differentiation of dendritic cells (Steinman, 1981; Witmer-Pack et al., 1987; Heufler et al., 1988; MacPherson et al., 1989; Markowicz and Englemen, 1990).

In vitro observations are supported by the study of mutant osteopetrotic *op/op* mice, which lack CSC-1 systemically (Wictor-Jedrzejczak et al., 1990) but produce normally GM-CSF, IL-1, IL-3, and IL-6 (Yoshida et al., 1990). In *op/op* mice the macrophage population is drastically decreased in all tissues, but to various degrees (Naito et al., 1991; Wictor-Jedrzejczak et al., 1992; Witmer-Pack et al., 1993). The dendritic cells, however, are unaffected by the mutation. They are present in *op/op* mice in all tissues in which they are normally found and in normal frequency (Takahashi et al., 1993).

In view of the above, Naito et al. (1991) and Takahashi et al. (1993) proposed that dendritic cells differentiate through a pathway distinct from that of the majority of macrophages (there is a subpopulation of macrophages that do not require CSF-1 for their differentiation), and that they must originate either from GM-CFCs directly or from some earlier hemopoietic precursor cells.

MONOCYTES AS PRECURSORS OF MICROGLIA

Microglia are considered to belong to the mononuclear phagocyte system because they express Fc and CR3 receptors, are capable of immune phagocytosis and may contain lysozyme, nonspecific esterase, and peroxidase in their cytoplasm, all basic requirements for inclusion of a mononuclear phagocytic cell in the mononuclear phagocyte system. Membership in the system implies that cells have common cell markers and/or functions similar to those of other members of the system and that they all have a common origin. It also implies cell origin from hemopoietic stem cells along the monoblast-monocyte lineage. The suggestion that microglia may have an origin other than from the hemopoietic stem cells, challenges one of the basic premises of the mononuclear phagocyte system.

The notion that microglia form from monocytes was proposed originally by Sántha and Juba (1933) and expanded on by Juba (1934). Since then the literature on the subject has become voluminous. Considerable confusion exists as to what constitutes a lineage; a practical definition of a cell lineage was given recently by Herzenberg and Kantor (1993) as "the set of cells deriving from distinct, relatively undifferentiated (unrearranged) progenitors that have at least a limited capacity for self-renewal and give rise to progeny that are committed to differentiate into cells distinguishable by particular functional or phenotypic characteristics." To define a cell lineage, its hierarchical arrangements, kinetics, and regulation of cell progression along the lineage at the molecular level must be demonstrated. In studying the lineage of monoblasts-monocytes-microglia the difficulty is to recognize various blood leukocytes that have infiltrated the CNS (Sedgwick et al., 1991). The first attempts to prove that microglia are, indeed, of monoblast-monocyte lineage used carbonated silver impregnation, vital dyes, colloidal carbon particles, incorporation of [^{3}H]TdR into the DNA as cell markers and morphological studies, light and electron microscopy. Although all this work was conceptually sound and seemed to support the hypothesis, it did not give direct proof that monocytes do indeed transform into microglia (Matsumoto and Ikuta, 1985; Matsumoto et al., 1985; Schelper and Adrian, 1986; De Groot et al., 1992; Lassmann et al., 1993).

Then followed the use of more sophisticated methods to identify cells by means of antibodies such as F4/80 (unknown antigen), Mac-1 or OX42 (CR3 receptor), antivault antibodies (large-size ribonuclear-protein particles) (Chugani et al., 1991), and ED-1 antibodies (macrophage functional-state antigen) and lectins, particularly *Griffonia simplicifolia* isolectin-B_4 (GSA I-B_4). The problem in using presently available antibodies is that the immunoreactivity expressed on microglia is shared with the same im-

munoreactivity on monocytes and macrophages. The exceptions are the lectins, which seem to have more specificity to ameboid and ramified microglia (Streit, 1990; Boya et al., 1991). Recently, a number of new antibodies have been developed, but they have not yet been sufficiently investigated (Esiri and McGee, 1986; Gehrmann and Kreutzberg, 1991; Flaris et al., 1993).

In spite of these difficulties, studies using antibodies and lectins pinpointed the timing and location of the appearance of monocytes-macrophages-microglia in the CNS in embryos, fetuses, and adult animals. The exact interphase between monocytes and microglia, however, still remains elusive and subject to the individual investigator's interpretation. Based on these studies, it is generally agreed that ramified cells in the parenchyma throughout the CNS are the "resting" form of microglia. It is also generally agreed that F4/80-positive cells found in the blood vessels and connective tissue of the leptomeninges, choroid plexus, and in Robin-Virchow spaces around the blood vessels in the CNS parenchyma are either monocytes or macrophages.

The vascularization of the CNS begins from the prominent perineural vascular plexus that surrounds the CNS. Vascularization has a caudal-cephalic gradient and commences at the myelencephalon and progressively ascends through metencephalon, mesencephalon, diencephalon, and telencephalon (Marin-Padilla, 1985). The capillary sprouts perforate the CNS and the penetration of the leading endothelial cells is achieved by focal degeneration and disintegration of subadjacent glial endfeet. Fragments of degenerating membranes and disintegrating processes are always found around the perforating endothelial cells (Marin-Padilla, 1985, 1988). The disintegrating cells attract monocytes and macrophages from the blood circulation.

No F4/80 positive cells have been found in the CNS before blood vessels begin to invade (Hume et al., 1983), although in the retina of rabbits nucleoside diphosphatase (NDPase) positive microglia appear at least 1 week before the onset of vascularization of the retina (Schnitzer, 1988, 1989). In mice, vascularization occurs between embryonic days 10 and 15, and at corresponding times in other animals (Strong, 1964; Bär and Wolff, 1972; Marin-Padilla, 1985, 1988). At embryonic day 10, F4/80 positive cells are seen in the leptomeninges (Andersen and Matthiessen, 1966; Morris et al., 1991; S. Fedoroff, I. Ahmed and G. Blevins, unpublished observations). During capillary invasion, single F4/80 positive cells are found in the CNS in the vicinity of capillaries. They are usually round or irregular in shape and may have some pseudopodia. The cytoplasm often appears vacuolated. The morphology of the F4/80 cells in the CNS is very similar to other F4/80 positive cells in the leptomeninges. The overall impression is that the F4/80 positive cells probably migrate into the CNS because of attraction by dead neural cells (Perry et al., 1985; Gordon et al., 1992; Ling and Wong, 1993).

Esiri et al. (1991) analyzed sections of necropsy brain tissue from fetuses and infants ranging in age from 18 weeks gestation to 8 months postterm. He found that fetuses of less than 28 weeks of gestation had a great concentration of macrophages in the ventricular zone (germinal matrix). They also saw macrophages in perivascular sites, leptomeninges, and subependymal regions. They observed fully developed microglia at 35 weeks of gestation. Hutchins et al. (1990) also observed great accumulations of ameboid cells with short processes in the ventricular zone (germinal matrix) of human fetuses at 13 to 24 weeks of gestation. They identified microglia positive for *Ricinus communis* agglutin-1 (RCA1) as early as 13 weeks of gestation.

At about embryonic days 15 to 16 in rats there is a greater influx of F4/80$^+$ cells into the CNS, and around birth, ameboid hemopoietic cells appear in large numbers in the corpus callosum (Imamoto and Leblond, 1978; Ling et al., 1980). The round, ameboid hemopoietic cells and Mac-1 positive cells gradually disappear during postnatal development (Imamoto and Leblond, 1978; Matsumoto and Ikuta, 1985; Ling and Wong, 1993).

In late embryonic development (embryonic days 18 to 19) in rats, some ramified F4/80$^+$ cells begin to appear in the parenchyma. They have an entirely different morphology than that of the F4/80$^+$ cells seen in earlier embryos (Perry et al., 1985). These cells have little cytoplasm but have several long processes that branch several times. The processes seem to be covered with very fine protrusions (Perry et al., 1985). It is generally agreed that these are ramified microglia. Their numbers gradually increase, then increase dramatically between postnatal days 5 and 15. By day 15 the entire parenchyma is filled with these ramified microglia, each one occupying its own territory with no overlapping. In different areas of the CNS, the density of the cells varies from 5 to 20% of cells (Kreutzberg, 1987; Lawson et al., 1990). There is no correlation, however, between the density of the ramified microglia and areas where there had been high cell death. The two phenomena appear to be independent (Lawson et al., 1990).

From these studies it becomes clear that hemopoietic macrophages are present and play a significant role in the developing nervous system. This is not surprising, since many dying cells and cell debris

have to be removed, hence the attraction of macrophages to the developing nervous system. However, as the system develops, the number of dying cells decreases and so does the number of hemopoietic macrophages, although some are always present in the CNS.

The development of microglia seems to be an independent process; they appear much later in development, have a specific developmental "window" when they proliferate, and eventually occupy the whole parenchyma of the CNS and, functionally, become closely integrated with the neurons and astroglia and probably also with the oligodendroglia (Fedoroff, 1990; Fedoroff et al., 1993).

A number of investigators who have extensively studied the invasion of macrophages into the CNS and the development of microglia conclude that no intermediate forms are present (Choi, 1981; Matsumoto and Ikuta, 1985; Matsumoto et al., 1985; Shelper and Adrian, 1986; De Groot et al., 1992). However, others do describe transitional forms (Imamoto and Leblond, 1978; Ling et al., 1980; Perry et al., 1985; Ling and Wong, 1993). Considering the great plasticity of microglia and how easily they acquire different phenotypic expressions, it may be very difficult to determine on morphological grounds whether a given morphological phenotype is due to cell modulation or to differentiation into a more advanced cell form.

TURNOVER OF MICROGLIA IN CENTRAL NERVOUS SYSTEM PARENCHYMA

The turnover rate of microglia has been studied using [^{3}H]TdR or [^{14}C]TdR incorporation and using bone marrow chimeras with genetic markers. Korr (1980) identified microglia using silver carbonate impregnation followed by autoradiography. When he injected mice with [^{14}C]TdR between postnatal days 10 and 14, he found that in 45-day-old mice about 50% of microglia were labeled. When the mice were injected between days 15 and 19, only 5% of microglia were labeled in 45-day-old animals. However, if the injections were made after day 20, no labeled microglia were found. Similar observations were made by Imamoto and Leblond (1978). When they injected [^{3}H]TdR into postnatal day 5 rats they found 78.1% labeled microglia in day 12 animals and 91% in day 19 animals. However, rats injected with [^{3}H]TdR on day 19 or day 20 had a significantly smaller number of labeled microglia (Imamoto et al., 1978) and when the injections were made after day 25, there were no labeled microglia.

The turnover of microglia in adult animals was studied by McCarthy and Leblond (1988) who identified microglia in semithin sections stained with toluidine blue. They infused [^{3}H]TdR into adult mice for 7 days and observed only one labeled pair of microglia. After 30 days of infusion only two labeled microglia were seen. They therefore concluded that in adult animals microglia normally do not divide (McCarthy and Leblond, 1988). Lawson et al. (1992), however, using F4/80 antibody to identify microglia, injected 3-month-old mice with methyl-1',2'[^{3}H]TdR and found that after 1 hour 0.05 to 0.06% of microglia were labeled; after 24 hours, 0.14 to 0.24%; and after 48 hours, 0.23 to 0.24%. They concluded that in adult animals microglia have a slow turnover rate.

From these observations it can be concluded that microglia develop primarily after birth and mainly between postnatal days 5 to 20, the time when microglia are proliferating rapidly, corresponding to actual observations of a tremendous increase in the number of microglia present in the parenchyma all through the CNS (Perry et al., 1985; Lawson et al., 1990; S. Fedoroff, unpublished results).

There is a discrepancy in findings pertaining to turnover of microglia in adult animals, probably due to different sampling methods used. Lawson et al. (1992) found a very slow turnover using immunocytochemistry. Others (Imamoto and Leblond, 1978; Korr, 1980; McCarthy and Leblond, 1988), using morphological methods, however, concluded that microglia do not divide in adult animals. It is possible that microglia are long-living, nondividing cells during steady-state conditions. It is also possible that microglia have a very long cell cycle; they have not lost the ability to divide, because in case of brain or spinal cord injury, they can suddenly proliferate. They also proliferate extensively in tissue culture in the presence of CSF-1.

The conclusions obtained with incorporation of [^{3}H]TdR are colored by the limitation of [^{3}H]TdR technology, which can reliably measure only cells that have short cell cycle times; cells with long cycle times are not detected by a [^{3}H]TdR assay. [^{3}H]TdR incorporation into the cell's DNA is possible only during the S phase of the cell cycle, when new DNA is synthesized. The S phase is generally relatively short, 6 to 12 hours. However, the G_1 phase of the cycle is variable, and if microglia have an exceptionally long G_1 phase, chances to incorporate [^{3}H]TdR during the S phase are meager. New technology utilizes protein specific to G_1 and G_0 phases of the cell cycle. Cyclins are found in the G_1 phase of the cell cycle of dividing cells (Waseem and Lane, 1990; Foley et al., 1991; Berlingin et al., 1992; Sasaki et al., 1992) and statin in the G_0 phase of nondividing cells

(Fedoroff et al., 1990; Shipper et al., 1992, 1993). Using antibodies to cyclins and statin it will be easier to determine whether microglia are in a cell cycle with a very long G_1 phase, or are differentiated cells in the G_0 phase.

Another approach to determination of cell turnover was to use radiation bone marrow chimeras. In these studies polymorphism of MHC class I antigens was used as a marker. Lewis rats express MHC class I type RT-1^l and DA rats express MHC class I type RT-1^a molecules. The bone marrow of the F_1 hybrids of these rats expresses MHC class I type RT-$1^{a/l}$. The bone marrow of such F_1 hybrid rats was injected into irradiated (1000 R) DA rats and the donor bone marrow progeny were identified with Il-69 monoclonal antibody, which immunoreacts with RT-1^l haplotype of the MHC class I molecule. The other type of bone marrow chimera was constructed by using F_1 hybrids of Lewis rats (MHC class I type RT-1^l) and PVG rats (MHC class I type RT-1^c) as bone marrow donors. The bone marrow of F_1 animals was injected into irradiated (900 R) Lewis rats. The OX27 monoclonal antibody recognizes c haplotype of MHC class I molecules. If microglia originate from the monoblast-monocyte lineage in the bone marrow, it would be expected that after a certain period of time the host's microglia would be replaced by new chimeric donor bone marrow cells carrying their specific MHC class I type haplotype. The chimeric CNS was analyzed with antibodies specific to donor type I MHC haplotype, 2 months up to more than 1 year after the reconstitution of the animals with marked donor bone marrow (Matsumoto and Fujiwara, 1987; Hickey and Kimura, 1988; Sedgwick et al., 1991; Hickey et al., 1992).

The results of all these studies with chimeric animals are mutually supportive, and there is general agreement that monocytes-macrophages in leptomeninges, choroid plexus and perivascular areas are indeed replaced by cells from the bone marrow. Within the leptomeninges, 60 to 70% of monocytes-macrophages are replaced by donor bone marrow cells, in choroid plexus, 20%, and in the perivascular area, 20 to 40%, during the 2 months after replacement of bone marrow.

In the CNS parenchyma, 5 to 10% of cells had donor bone marrow cells with MHC class I type markers. These were small round cells with prominent nuclei, a thin rim of cytoplasm and short processes. The cells were located either free in the Virchow-Robin space or situated along capillaries or small vessels between the basement membrane of the endothelial cells and the astroglial foot processes of the glia limitans (Hickey and Kimura, 1988; Graeber et al., 1989; Lassmann et al., 1991; Hickey et al., 1992). These cells were identified as lymphocytes. Only a very few parenchymal ramified microglialike cells expressed donor MHC class I haplotype (Matsumoto and Fujiwara, 1987; Hickey et al., 1992). Bone marrow chimeras that remained chimeric for periods beyond 1 year past marrow transfer still exhibited only scattered, rare, ramified microglialike cells expressing donor bone marrow MHC class I haplotype (Sedgwick et al., 1991; Hickey et al., 1992).

To stimulate turnover of bone marrow-derived cells in the CNS, Lassmann et al. (1993) developed experimental allergic encephalomyelitis (EAE) in rats with radiation bone marrow chimeras by injecting intravenously myelin basic protein reactive T-lymphoblasts. They analyzed the CNS of these rats at the peak of the disease and 3 months after recovery from inflammation. In recovered animals, 90% of the cells in the meninges had donor bone marrow MHC class I haplotype; 4.4% in the perivascular space; and 5.6% in the parenchyma of the CNS. Most donor bone marrow MHC class I haplotype positive cells in the parenchyma were small and round, without processes. They were positive for antigens characteristic to T lymphocytes. Only 0.6% of all donor bone marrow MHC class I haplotype positive cells had morphology characteristic to ramified microglia. They concluded that the ramified microglia, resident in the parenchyma of the CNS, are extremely sessile, even in pathological conditions, and are not replaced by bone marrow-derived marked cells in any significant numbers (Lassmann et al., 1993).

Sedgwick et al. (1991) induced inflammation in the CNS of bone marrow chimeric rats either by intracellular inoculation with the neurotrophic murine hepatitis virus strain JHM or by subcutaneous injection of myelin basic protein. After 7 days, the rats were killed and the microglia and hemopoietic cells present in the CNS were isolated by first disaggregating the cells by passing through a stainless steel sieve, and then by collagenase. The cells were then separated on a Percoll gradient and immunoreacted with a number of antibodies. The immunoreacted cell populations were analyzed by cytofluorographic analysis using an FACScan. With this procedure the microglia could be distinguished from the infiltrating lymphocytes, primarily by the degree of expression of CD45 panhemopoietic cell antigen. In such preparations microglia expressed the host's MHC class I haplotype molecules but not the donor type (Sedgwick et al., 1991). Even after one year of the chimeric state the microglia did not change (J.D. Sedgwick, personal communication).

In all the above experiments polymorphism of

MHC class I molecules was exploited to identify cells. The problem is that not all cells express MHC class I molecules to the same degree (Wong et al., 1984; Perry and Gordon, 1988); therefore, before analysis the animals had to be injected with murine gamma-interferon (INFγ) and murine tumor necrosis factor-alpha (TNFα) to upregulate the expression of MHC class I antigen. There is some probability that not all cells respond to upregulating of the MHC class I molecules to the same degree.

De Groot et al. (1992), therefore, used bacteriophage λ transgenic mice as bone marrow donors. Bacteriophage λ is incorporated into the genome of the transgenic animals and therefore, in contrast to the protein markers, it is a very stable marker and is not subject to the posttranslational or posttranscriptional modification. They transplanted the bone marrow (from transgenic mice) into recipient 1-day-old or adult (3-month-old) mice. Integrated copies of λ DNA donor cells were detected by *in situ* hybridization, and the types of cells were identified by prior immunocytochemistry. They found, as others had, many donor bone marrow-derived cells in the leptomeninges, ventricles, and occasionally in blood vessel walls in the brain parenchyma. These cells resembled macrophages; they had round or ovoid nuclei and extensive cytoplasm and were devoid of processes. They also found approximately 10% of microglialike cells that displayed the donor transgenic signal in the white matter of the cerebellum, medulla oblongata, and corpus callosum. No microglialike cells having the donor transgenic signal were found in the gray matter. De Groot et al. (1992) concluded that microglia constitute a heterogeneous cell population and that only a small portion are derived from newly recruited bone marrow precursors, whereas the majority of the microglial cells must have come from locally present precursors, presumably of neuroectodermal origin.

From the experiments using isotope incorporation into DNA and those using chimeric animals, it can be concluded either that microglia are long-living cells with an extremely low turnover rate or that their origin and turnover rate during adult life does not depend on infiltration of cells from the bone marrow. Another possibility is that the CNS is colonized by some of the hemopoietic stem cells during the early stages of embryonic development and that the stem cells become resident cells of the CNS, responsible for formation and regular turnover of microglia during adult steady-state conditions, and having the ability to respond in pathological conditions where increased numbers of microglia are required (Sedgwick et al., 1991). If such hemopoietic stem cells are indeed present in the CNS, they must retain their ability to self-replicate to maintain a constant microglia cell population under steady-state conditions. At the same time, the neural microenvironment must have restricted these stem cells from forming other hemopoietic cells, as such cells are not seen in the CNS. In addition, the phenotype of monoblast-monocyte precursor cells would have to be modified from that in the bone marrow, because in adult brain, monoblasts-monocytes, which in this possibility would be the precursors of microglia, are not seen in the parenchyma to the extent they should be if they are indeed the precursors of microglia during development and adult life.

For purposes of an overview, it is important to mention that in all the above studies many small hemopoietic cells were found in the CNS parenchyma and were identified as lymphocytes (Lassman et al., 1991; Sedgwick et al., 1991; De Groot et al., 1992). It has been proposed that the CNS is continuously patrolled by a small number of T lymphocytes and monocytes-macrophages. However, they do not remain in the CNS for a long time, probably 1 or 2 days (Hickey et al., 1991), although there is evidence that some T lymphocytes can survive in the CNS for several months (Sedgwick et al., 1991). In the presence of antigens the "patrolling" T lymphocytes and macrophages can remain in the CNS, or cyclically reenter to initiate inflammation (Ferrer et al., 1990; Hickey et al., 1991).

HEMOPOIETIC STEM CELLS IN THE CENTRAL NERVOUS SYSTEM

Techniques to detect and measure the number of hemopoietic stem cells within hemopoietic tissue are well established. To detect self-replicating multipotential stem cells, mice are irradiated (1000 R) and dilutions of hemopoietic cells are injected into the mouse's tail vein. The multipotential stem cells present in the injected sample home in the spleen and form clones. By analyzing the composition of the clones, the type of stem cell that gave rise to them can be identified and by counting the number of clones (colonies) found in the spleen, the number of stem cells present in the injected sample can be calculated (Metcalf, 1984).

If, during embryogenesis, the CNS is colonized by hemopoietic stem cells, then they should be detectable by a spleen colony assay. Bartlett (1982) disaggregated whole brains from (CBA X BALB)F_1 mice and injected cells into irradiated (850 R) CBA mice. After 9 days, he examined the spleens and found 14 spleen cell colonies per 10^5 cells injected into adult animals, and 8.9 spleen cell colonies per

10^5 injected cells in 3-day-old animals. He also used disaggregated cell suspensions from the brains of W^f/W^f mice to successfully cure congenital anemia in W^f/W^f mice when transplanted cells from bone marrow of W^s/W^s mice were unable to do so. He concluded that mouse brain does, indeed, have pluripotential hemopoietic stem cells that probably colonize the CNS during early embryogenesis and that in the appropriate environment (spleen) they are capable of giving rise to various hemopoietic cells.

Hoogerbrugge et al. (1985) was unable to obtain the same results when he repeated Bartlett's experiments. They could not find hemopoietic stem cells in the brains of W/W^v mice and injection of disaggregated brain cells into three different strains of mice yielded only 2.6 to 5.7 spleen cell colonies per brain. However, when the brains were carefully washed in balanced Hanks' solution following dissection, the number of spleen colonies in the irradiated host animals decreased to 0.1 to 0.4 colonies per brain. They could also recover hemopoietic stem cells from the wash fluid. They concluded that most of the cells in Bartlett's preparations must have been derived from contaminating bone marrow and blood (Hoogerbrugge et al., 1985).

In collaboration with Dr. A.A. Axelrad, University of Toronto, we obtained results similar to those of Hoogerbrugge et al. (1985). We used newborn C_3H/HeJ mice as a source of neopallial cells and injected the cells into irradiated (925 R) 10-week-old (C_3Hf/Bi X C_{57} B1/6)F_1 hybrid mice. We obtained 2.3 spleen colonies per 10^6 injected cells. We also injected cells from tissue cultures initiated from newborn C_3H/HeJ mouse neopallia, which formed microglialike cells. (For more detail, see below). The injection of 10^6 cultured neopallial cells yielded on the average 1.7 spleen colonies. When we analyzed the colonies formed by injecting neopallial cells and tissue culture cells, all cells had the Y chromosome corresponding to the male sex chromosome of the recipient mice and one "marker" chromosome of the recipient mice, indicating that the spleen colonies were of host radiation residual stem cell origin and not from transplanted cells (Hao et al., 1991).

In addition, we grew cells of neopallial cell tissue cultures in methylcellulose cultures which are used to detect granulocyte-macrophage colony-forming cells (Guilbert and Iscove, 1976; Iscove et al., 1982; Metcalf, 1984). After 16 days of incubation with and without monocyte-macrophage growth factors (IL-3 and CSF-1), no proliferation of cells could be detected, indicating that no monocyte-macrophage precursor cells were present in cultures.

From the experiments of Hoogerbrugge et al. (1985) and our experiments (Hao et al., 1991), we concluded that mouse brains do not contain hemopoietic stem or precursor cells that are detectable by the classical spleen colony assay (Till and McCulloch, 1961), or by methylcellulose cultures (Guilbert and Iscove, 1976; Iscove et al., 1982; Metcalf, 1984).

ORIGIN OF MICROGLIA IN TISSUE CULTURE

Hao et al. (1991) demonstrated that when neopallial cell cultures are subjected to nutritional deprivation (i.e., no medium change), microglia develop preferentially. The explanation was that during the period of nutritional deprivation when the culture medium is not changed, microglial trophic factors, including CSF-1, secreted by astroglia, accumulate in a concentration sufficient to stimulate microglial differentiation. Nutritional deprivation is therefore the method of choice to produce microglia in cultures and to study their differentiation (Hao et al., 1991; Richardson et al., 1993; Fedoroff et al., 1993; Neuhaus and Fedoroff, 1994). To engender fuller appreciation of these observations I will describe the culture system used.

The cultures are usually initiated from neopallia of newborn C_3H/HeJ (endotoxin resistant) mice. After 8 to 10 days in culture, a monolayer of cells forms. In dense cultures, better than 95% of the cells are GFAP-positive, indicating that they belong to the astroglia lineage. Usually less than 1% of the cells express macrophage-specific antigens such as Mac-1 or F4/80, and these cells can be eliminated from the culture by complement-mediated immune-cytotoxicity (Hao et al., 1991). When the medium is no longer changed, pleomorphic phase-dark cells appear on the surface of the layer of astroglia. The astroglia begin to retract from the plastic substratum, providing free space for the newly formed cells to attach to the plastic. When they adhere to the plastic surface they assume typical ameboid cell morphology and develop a number of vacuoles in their cytoplasm. These cells are highly motile. After 10 to 12 days of nutritional deprivation, the whole culture is populated with the ameboid microglia. At the same time, the astrocytes retract from the substratum and form clumps that can easily be washed out from the cultures. Thus, in conditions of nutritional deprivation, in a period of 8 to 10 days, the monolayer culture initiated from the neopallia of newborn mice completely transforms into a culture of microglia (Hao et al., 1991, 1992).

In cultures, microglia can assume several different morphological forms, for example, ameboid, ramified, rod-shaped, round-flat shape, and spindle shape

(Jordan and Thomas, 1988; Hao et al., 1990; Suzumura et al., 1991; Ward et al., 1991; Abd-El-Basset and Fedoroff, 1995). Microglia in neopallial cell cultures have typical macrophage ultrastructure. They can mediate Fc-immune phagocytosis and secrete lysozyme into the medium. They express vimentin, Mac-1, Mac-3, F4/80, Fc, CR3, LC-1, and MHC class II antigens, take up Dil-ac-LDL and contain nonspecific esterase. They differ from macrophages of bone marrow, spleen, and peritoneal exudate in that they do not attach firmly to the plastic substratum (Hao et al., 1991).

We were able to generate microglia in cultures initiated from every stage of mouse embryonic development, beginning with Theiler stage 13 (8.5 days of gestation). Thus the formation of microglia in culture is not related to any specific developmental stage (Hao et al., 1991). It follows that microglia precursor cells must be present throughout the developing CNS and in cultures these cells are neither morphologically nor immunologically distinguishable from astroglia precursor cells, that is, they are vimentin$^+$, GFAP$^-$, Mac-1$^-$, and F4/80$^-$. To determine the number of precursor cells present in a culture initiated from neopallia of newborn animals, we used a limiting dilution analysis and found that one in every 8 cells (12%) in cultures is a potential microglia precursor cell (Richardson et al., 1993). Twofold serial dilutions (up to 50 cells per culture well) of disaggregated neopallial cells, in the presence of trophic factors, generated in cultures at every dilution point approximately the same frequency of microglia or microglia-containing cell colonies, indicating that the microglia formation was independent of dilution of neopallial cells (number of neopallial cells planted per culture).

Using nutritionally deprived neopallial cell cultures we unexpectedly found that at the time the numbers of microglia were increasing rapidly in the cultures, no cell divisions could be detected. When we triple labeled the cultures for Mac-1, GFAP, and BrdU, no Mac-1$^+$/BrdU$^+$ cells could be found, indicating that no microglia progenitor cells divided. BrdU$^+$ cells (dividing cells) that were present were always either GFAP$^+$ or GFAP$^-$. Infecting the cultures with a retrovirus having a marker lacZ reporter gene did not produce microglia that had the marker (Neuhaus and Fedoroff, 1994).

It should be stated, however, that in cultures that receive microglial growth factors IL-3, IL-6, and CSF-1 at the time of regular medium change, microglia form equally well and proliferate. The massive cell death that occurs in cultures subjected to nutritional deprivation does not take place (Richardson et al., 1994). Therefore the extensive cell death is not a factor in microglia formation in cultures. They require appropriate growth factors and require these at certain concentrations and in certain combinations, in order to differentiate (Richardson et al., 1994).

This indicates that microglia progenitor cells in neopallial cell cultures that are subjected to nutritional deprivation are in a quiescent state and that in the presence of appropriate trophic factors, secreted by astroglia, they transform into Mac-1$^+$ microglia directly, without cell division. Once they have transformed, then in the presence of CSF-1 (macrophage growth factor) they proliferate and expand the population of Mac-1$^+$ microglia in the culture (Neuhaus and Fedoroff, 1994). Alliot et al. (1991) also described the presence of microglia progenitor cells in astroglia cultures. In their cultures the progenitor cells were Mac-1$^+$/F4/80$^-$ and on culturing they became Mac-1$^+$/F4/80$^+$. These progenitor cells proliferated vigorously when seeded on an astroglia feeder layer. It seems that the microglia progenitor cells described by Alliot et al. (1991) have progressed further along the lineage of cell differentiation than the ones we describe and that they could proliferate on top of astroglia because astroglia constitutively secrete macrophage growth factor CSF-1.

Where do the microglia precursor cells come from? I have mentioned that we were able to develop microglia in cultures initiated at any developmental stage. To find out whether neuroectoderm is the source of microglia precursor cells, we initiated cultures from neuroepithelium dissected carefully from C_3H/HeJ mouse embryos at Theiler stage 13 (day 8.5 of gestation). At this developmental stage the neuroepithelium of the neural tube is not vascularized and monocyte-macrophage cells have not yet begun to appear either in the yolk sac or elsewhere in the embryo. To minimize the possibility that some mesodermal cells might still be attached to the neuroepithelium, it was treated lightly with a trypsin solution to dislodge any mesodermal cells still attached. The neuroepithelium was cut into small fragments and grown in cultures for 20 days, with regular feeding every 2 to 3 days. After 20 days, the medium was not changed again, and after an additional 10 to 14 days in such nutritionally deprived culture conditions, many microglia appeared. These experiments strongly suggest that microglia can originate from neuroepithelium as do other glia.

It is possible that microglia develop from glioblasts and are closely related to the astroglia lineage, from which they may branch off around the time of birth, when high rates of microglia proliferation have been observed (Imamoto and Leblond, 1978;

Korr, 1980) or, possibly, branch off earlier in development but remain quiescent until birth. Microglia and astroglia are closely related functionally (Fedoroff et al., 1993). Astroglia secrete CSF-1, the growth factor for microglia which require it for survival, proliferation, and differentiation (Fedoroff et al., 1993). However, microglia seem to be very plastic and CSF-1 does not seem to be always essential for their differentiation.

Mice homozygous for the *op* mutation, as mentioned before, have severe osteopetrosis, because of their lack of osteoclasts (which form from macrophages). The recessive osteopetrotic *op* mutation in mice results in a total lack of CSF-1 in body fluids and tissues (Wictor-Jedrzejczak et al., 1990; Yoshida et al., 1990), but production of the other cytokines, GM-CSF and IL-3, which are involved in mononuclear phagocyte cell differentiation, is unaffected or even increased (Wictor-Jedrzejczak et al., 1990). Mononuclear phagocytes in *op/op* mice are depleted to varying extents in different tissues (Naito et al., 1991; Wictor-Jedrzejczak et al., 1992; Witmer-Pack et al., 1993). However, Naito et al. (1991), Takahashi et al. (1993), Witmer-Pack et al. (1993), and Blevins and Fedoroff (1995) reported that microglia are not depleted in the brains of *op/op* mice. The frequency and morphology of microglia in *op/op* mice in various parts of the brain are similar to those in their littermates (+/?) or mice of other strains that normally produce CSF-1 (Blevins and Fedoroff, 1995).

In our experiments the neopallial cells from newborn *op/op* mice in cultures without medium change did not form microglia and the astroglia did not secrete CSF-1; whereas the neopallial cells from their +/? littermates in cultures grown without medium change did form microglia and the astroglia did secrete CSF-1 into the medium. When we added CSF-1 to the cultures of neopallial cells from newborn *op/op* mice, Mac-1 positive microglia formed (Blevins and Fedoroff, 1995). This indicated that microglia precursor cells are present in the newborn *op/op* mice, and that they require CSF-1 for development in cultures. *In situ,* however, in *op/op* mice microglia can form in the absence of CSF-1. It is likely that factor(s) which substitute for CSF-1 *in situ* in *op/op* mice are produced locally (Blevins and Fedoroff, 1993).

There are certain similarities between microglia in the CNS and Langerhans cells in the epidermis. Both are antigen-presenting, both express CR3, Fc, and Ia receptors (Fossum, 1989; Perry and Gordon, 1991) and their numbers are not affected in *op/op* mice (which are deficient in CSF-1). The difference between the two cell types is that Langerhans cells in cultures depend for viability and function on GM-CSF, but microglia depend on CSF-1 (Witmer-Pack et al., 1987; Heufler et al., 1988; Fedoroff et al., 1993, Blevins and Fedoroff, 1995). The numbers of Langerhans cells in the epidermis of *op/op* mice and the numbers of microglia in the CNS of such animals are unaffected by the mutation, but for different reasons. Langerhans cells develop normally because in *op/op* mice the production of GM-CSF is unimpaired. Microglia, however, develop normally in spite of the total absence of CSF-1, probably by utilizing an alternate pathway of differentiation that does not include CSF-1.

The observations made on tissue cultures indicate that microglia can be produced by neuroepithelial cells and that the neopallia of newborn animals have cells that are Mac-1 negative which under appropriate culture conditions can form microglia. The studies of osteopetrotic mutant *op/op* mice indicate that microglia differ from dendritic cells and that both microglia and dendritic cells differ from classical macrophages.

ORIGIN OF MICROGLIA *IN SITU*

Recently McKanna (1992, 1993a, 1993b), using immunocytochemistry, showed that in rats microglia and their progenitor cells are immunoreactive for lipocortin-1 (LC-1). By using this "marker" he could pinpoint the glia raphe of the floor plate of the hindbrain and spinal cord as the site of origin of microglia. Because of the significance of this observation, I will expand on the development of the floor plate of the neural tube.

During the gastrulation stage of development the bilaminar embryonic disc is converted into a trilaminar disc. This stage is marked by the epiblastic cells (from which all three germ layers develop) beginning to converge in the midline of the dorsal aspect of the embryonic disc. They form a linear band, the primitive streak, which invaginates. The cells in the primitive streak lose their attachment to the epiblastic cells and migrate laterally between the epiblast (future ectoderm) and hypoblast (future entoderm), thus forming a loose network of mesoblast, which forms the intraembryonic mesoderm.

At the cranial end of the primitive streak, cells accumulate and form the primitive knot and a pit from whose rostral lip the cells segregate. Some form the notochord which grows in the midline between ectoderm (former epiblast) and entoderm (former hypoblast). However, cell marking experiments have shown that other cells of the rostral lip of the primitive pit migrate rostrally in the midline above the

notochord and form the floor plate (notoplate) of the neural tube (Jacobson and Gordon, 1976).

Whether or not the cells in the primitive knot are already determined as becoming neuroepithelial cells that form the floor plate (notoplate) or becoming cells that form the notochord, is not known. In amphibians they are segregated, but both form from the same blastomere (Jessell et al., 1989). Thus there is a view that cells of the notochord and the cells of the floor plate (notoplate) of the neural tube have a common ancestry. This view, however, has not yet been generally accepted (Jessell et al., 1989; McKanna, 1993b).

The cells of the floor plate differentiate early in embryogenesis to form a glial raphe as the lateral parts of the neural plate begin to fold into a neural tube (Jessell et al., 1989; McKanna, 1993b). The cells of the raphe are primitive glial ependymal (PGE) cells which form the midline axis. This axis subdivides the ventral part of the pons and spinal cord into two symmetrical compartments. The raphe spans through the hindbrain and spinal cord.

In transverse sections of E16.5 rat embryos, the LC-1 raphe forms in the median one third of the floor plate. It consists of pseudostratified epithelial cells, the primitive (archetypic) glial ependymal (PGE) cells, which span the whole thickness from the fourth ventricle in the pons, or the central canal in the spinal cord, to the pia, a distance in the pons of approximately 500 μm and in the spinal cord, 150 μm. The width of the LC-1 raphe in paraffin sections is approximately 20 to 30 μm, and it consists of 5 to 8 PGE cells with their nucleus in the apex and their long thin process distal toward the pia.

It is these PGE cells that stain positively with LC-1. The first staining appears in the rhombencephelon at E11 and reaches the lumbar cord by the end of E12. Staining of the raphe is maximal from E15 to E18 and subsequently declines (McKanna and Cohen, 1989; McKanna, 1993b).

LC-1 is a 35 kD Ca^{2+}-phospholipid binding protein that serves as a substrate for the EGF receptor tyrosine kinase (Fava and Cohen, 1984; McKanna, 1993b). It is also known as p35 or annexin I. LP-1 is a soluble intracellular protein, but it is also secreted by the cells. Extracellularly, it has an anti-inflammatory effect (Goulding and Guyre, 1992).

At the end of the first postnatal week, small LC-1-positive cells appear laterally from the LC-1-positive streak in the glial raphe. Their numbers increase and the most distally located LC-1-positive cells are ramified. By the fourth postnatal week a large number of LC-1-positive cells accumulate in the vicinity of the raphe and the raphe itself begins to decline. Some of the LC-1-positive cells have ameboid and some ramified morphology. By the sixth postnatal week and through adulthood, the LC-1-positive cells are present throughout the CNS in both the white and gray matter (McKanna, 1993b). The LC-1 positive cells are negative for S-100β, the marker specific for astroglia, but stain positively with HRP-labeled *Griffonia simplicifolia* isolectin-B_4(GSA I-B_4) which is used to demonstrate microglia (McKanna, 1993a, 1993b). The LC-1-positive ramified cells react to stab wounds or cortical ablation by increasing in number, acquiring ameboid morphology and becoming phagocytic (McKanna, 1993a).

In the previous section the formation of a highly enriched population of microglia in cultures was described. These microglia also immunoreact positively for LC-1 (Fedoroff and McKanna, 1994). Thus it seems likely that the LC-1-positive dendritic cells described by McKanna (1992; 1993a, 1993b) are indeed microglia and that they form from the PGE cells in the raphe of the floor plate of the hindbrain and spinal cord.

It is also significant, in this connection, that in the floor plate on either side of the LC-1-positive raphe, there are paramedian bands of cells that immunoreact with S-100β (McKanna, 1993b). McKanna followed these S-100β-positive cells and observed their lateral movement as development proceeded and that later they populated the CNS. The S-100β-positive cells in the CNS eventually had an astroglial morphology and immunoreacted positively for GFAP. Thus, both microglia and at least some astroglia form from PGE cells in the raphe of the floor plate of the neural tube (Figure 10-1).

CONCLUSIONS

Hemopoietic monocytes-macrophages are present in the CNS during most of development and their presence is related to neural cell death. They occur in large numbers throughout embryonic development, then decrease and finally are limited to a few cells that "patrol" the CNS. Mature microglia, on the other hand, are not present during the early stages of embryonic development. In mice and rats, they form in large numbers during the post-natal period and occupy the parenchyma throughout the CNS. Their density and distribution in the parenchyma of the CNS is not related to sites of neural cell death during embryonic development. Their numbers are constant in the steady-state condition during adult life. Microglia are part of the neuronal-glial network and thus are functionally closely integrated with other neural cells.

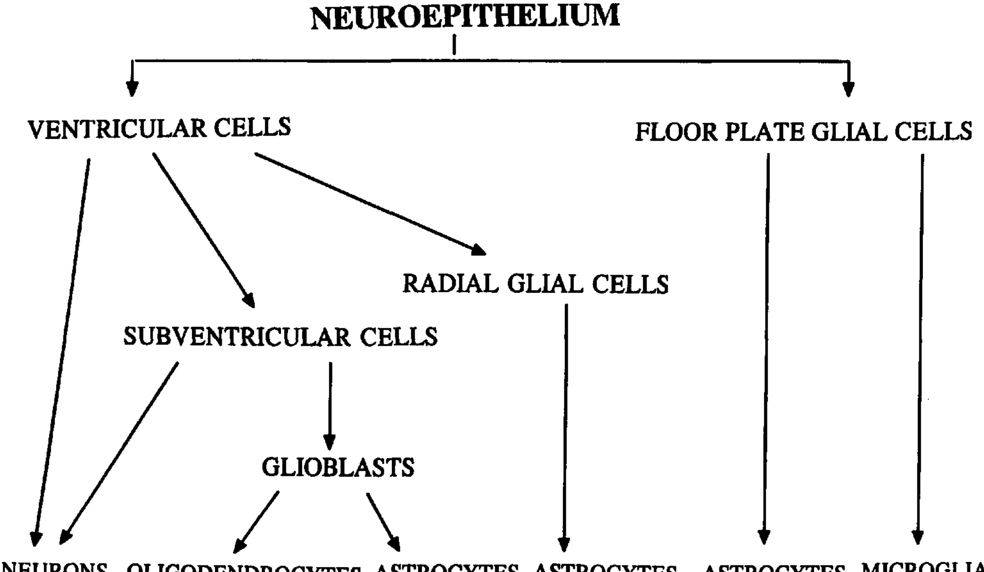

FIG. 10-1. Hypothetical scheme of gliogenesis. The proposal for glial development via ventricular and subventricular cells and glioblasts, and neuron development from ventricular and subventricular cells, was originally proposed by the Boulder committee (1970) (reviewed by Fedoroff, 1986; Levison and Goldman, 1993). Development of astrocytes from radial glia had already been proposed by Ramón y Cajal (reviewed by Schmechel and Rakic, 1979; Cameron and Rakic, 1991; Misson et al., 1991; Yang et al., 1993; see also Chapter 8, this volume). There is some evidence supporting the notion that oligodendroglia also develop in some parts of the CNS from radial glial cells (e.g., Hirano and Goldman, 1988). The development of the floor plate of the neural tube and the origin of microglia and astroglia from the glial raphe of the floor plate is described in the text. Oligodendrocytes may also originate in the ventral part of the spinal cord, but it is not clear whether they develop only from radial glia or also from primitive glial ependymal cells (Noll and Miller, 1993). It is also possible that some microglia develop via ventricular and subventricular cells and glioblasts, although the available data is not adequate (see text). A number of lineage choices appear to be available for glia in different regions of the CNS. Immature glia are motile and populate the CNS from the sites of their origin (see text). It is not known whether various glia cells for example, astroglia, that develop along different pathways, are identical or retain characteristics related to their lineage.

Attempts to demonstrate that microglia can be replaced in chimeric animals by marked monocytes-macrophages have been unsuccessful. In some cases a small number of microglialike cells had hemopoietic cell markers, but, considering the tremendous morphological plasticity of macrophages, it is not surprising that some could assume a morphology resembling that of microglia. Although microglia and macrophages share some common antigens and even functions, such as phagocytosis, this does not imply common origin. Searches for hemopoietic stem cells in the CNS have been unsuccessful. The recent findings that microglia originate from LC-1-positive primitive glial ependymal cells in the raphe of the floor plate of the hindbrain and spinal cord and that neuroepithelium taken from embryos at stages of development before vascularization of the CNS has occurred could generate microglia in cultures, strongly support the notion that microglia are of neuroectodermal origin as are all other glia (Figure 10-1), and that microglia are an integral part of the CNS fabric.

It is significant that microglia as well as at least some astroglia form from the primitive glial ependymal cells of the floor plate of the neural tube. Microglia and astroglia are closely linked functionally and microglia depend on CSF-1 secretion from astroglia. That they may also be closely linked developmentally does not seem impossible. Microglia express many phenotypic markers that are also expressed by hemopoietic mononuclear cells. Perhaps this is due to a common ancestry in developmental stages, even preceding development of the neuroectoderm and mesoderm.

On the whole, it seems unlikely that microglia form from hemopoietic cells. For over 70 years, research has been directed mainly to confirm the early claims that microglia do originate from pial or hemopoietic cells. Perhaps it is time to redirect the emphasis of the research and to concentrate on microglia as derivatives of neuroepithelium as are the other glia.

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III Physiology

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11 Potassium channels

STEVEN DUFFY, DOUGLAS D. FRASER, AND BRIAN A. MACVICAR

The analysis of the electrophysiological properties of glial cells is inspired by a desire to discover the functional roles of these cells in the intact nervous system. The output of neurons is easily documented and conceptualized (i.e., increased firing of action potentials or secretion of neurotransmitters) but except for myelination the roles for glia in the nervous system have still not been rigorously described. The close spatial relationship between glial cells and neurons suggests that glia may be important modulators of neuronal function. The proximity of glial endfeet to capillaries suggests that they may mediate the flow of nutrients to neurons. Although there is no obvious electrophysiological output from these cells, there is mounting evidence for the secretion of important factors such as growth factors or eicosanoids from glia. In our present conceptual framework for glia it is difficult to ascertain a functional role for voltage-gated ion channels. However, there is no doubt that glia express a diversity of ion channels approaching that of neurons. Elsewhere in this book inward Na^+ and Ca^{2+} channels and ligand-gated channels are discussed. Here we focus on the K^+ currents, which are the most predominant electrophysiological characteristics of glial cells.

The first electrophysiological recordings from glial cells were obtained in invertebrate preparations (squid: Villegas et al., 1963; leech: Kuffler and Potter, 1964). The characteristics of these cells are typical of glial cells that have been studied in many *in vivo* and *in vitro* preparations (Trachtenburg and Pollen, 1970; Ransom and Goldring, 1973; Takato and Goldring, 1979; Gutnick et al., 1981; Burnard et al., 1990). In comparison to neurons, glial cells have a more negative resting membrane potential (E_m), a lower input resistance, and a faster time constant. The lower resting potential results from a higher selective potassium permeability of glial cell membranes compared to neurons. Glial cells also do not typically display action potentials or other regenerative potentials. It should be kept in mind that, although glia express inward Na^+ and Ca^{2+} currents, they appear passive during intracellular recordings when no pharmacological manipulations are used to decrease K^+ permeability (but see Sontheimer et al., 1992 for an exception).

The advent of patch-clamping techniques and preparations such as acutely isolated cells have revealed a greater complexity of glial membrane properties than previously suspected from intracellular recordings. This chapter reviews the evidence for multiple types of K^+ channels in glial and where possible provides a conceptual framework for a functional role.

ASTROCYTES

Historical Perspective

To date, astrocytes in culture have been the most widely used preparation to identify and characterize voltage-dependent channels. The advantages of culture preparations are evident: the cellular phenotype is relatively homogeneous, they can be maintained for long periods, there is no diffusion barrier and the cells are more amenable to voltage-clamping using whole-cell patch-clamping techniques. There are some disadvantages to only studying cells in culture because culture conditions influence cellular phenotype (Raff et al., 1983; Juurlink and Hertz, 1985), including channel expression (Barres et al., 1989). To circumvent these problems, techniques have been developed that allow fast and reliable acute isolation of astrocytes from mature tissue (Newman, 1985; Barres, 1992; MacVicar et al., 1992; Tse et al., 1992) (Figure 11-1). Alternatively, ion channels have recently been studied *in situ* using whole-cell patch-clamp techniques, which *allow* a low access resistance and reliable intracellular dialysis (Clark and Mobbs, 1992; Steinhäuser et al., 1992; Sontheimer and Waxman, 1993).

Astrocytes have been classified into two groups: (1) fibrous, which are commonly localized to white matter, and (2) protoplasmic, which are commonly found in gray matter (Peters et al., 1976). In cell culture, fibrous astrocytes are process-bearing while protoplasmic are flat and polygonal. Both types contain glial fibrillary acidic protein (GFAP). However,

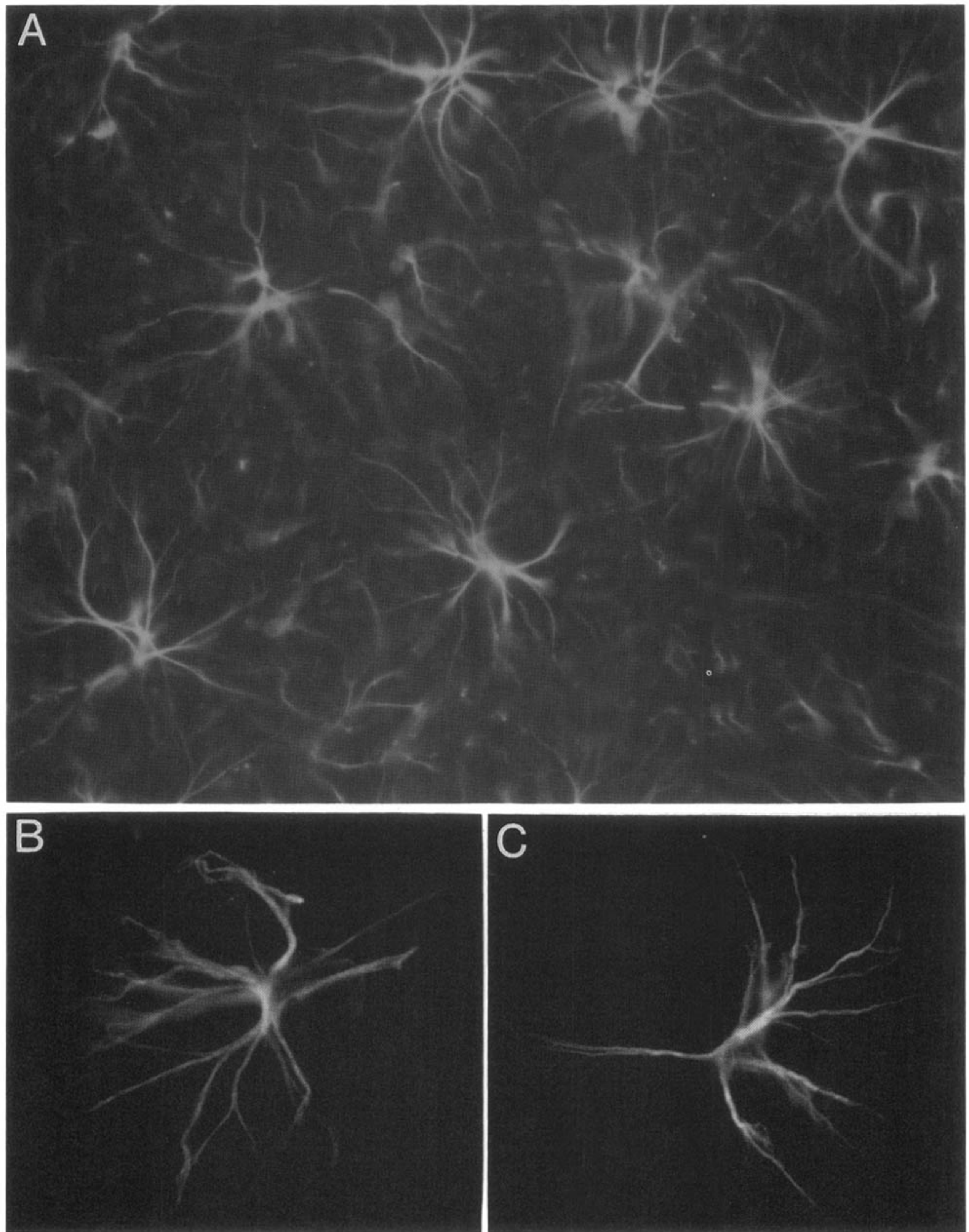

FIG. 11-1. Morphology of astrocytes immunohistochemically stained for glial fibrillary acidic protein (GFAP): Comparison between cells in the slice preparation or acutely isolated. (**A**) Astrocytes that were stained for GFAP in hippocampal slices after fixation and sectioning on a cryostat exhibited the typical stellate morphology. (**B, C**) Astrocytes acutely isolated using the technique described in MacVicar et al. (1992) still showed stellate morphology with good preservation of processes. [The photomicrograph in Figure A was supplied by Dr. L. Mudrick-Donnon.]

classification of astrocytes into either category may be difficult in several brain areas, including the hippocampus (Bignami et al., 1972). In cultures derived from the optic nerve, two astrocyte subtypes have been identified and termed type 1 and type 2 (reviewed by Barres et al., 1990c; Sontheimer, 1992). Type 2 are fibrous-like cells, which stain for both GFAP and the surface antigen termed A2B5, whereas type 1 are protoplasmic-like cells that are GFAP-positive and A2B5-negative. However, A2B5 staining of type 2 astrocytes has not been demonstrated *in vivo* in the optic nerve (Sontheimer, 1992). Thus, only where appropriate will we refer to protoplasmic as type 1 and fibrous astrocytes as type 2. Astrocytes obtained from acute isolation or in cultures devoid of A2B5 antigenicity will simply be designated by the brain region from which they were acquired.

Delayed Rectifier Potassium Channel

The delayed rectifier K^+ current (I_{DR}) was first described in the squid giant axon by Hodgkin and Huxley (1952; see Hille, 1992). This current is present in most excitable cells, and is primarily responsible for repolarization following spike discharge. Evidence for the expression of this channel in cultured astrocytes was obtained using whole-cell voltage-clamp techniques (Bevan and Raff, 1985; Bevan et al., 1985, 1987; Nowack et al., 1987; Barres et al., 1988, 1990a, 1990b; Sontheimer et al., 1992) (Figure 11-2). As in other cell types, the current is inactive at resting membrane potentials and is activated by depolarization. The activation threshold is typically -40 mV, and the conductance increase follows a sigmoidal time-course. Inactivation is minimal with maintained depolarization and deactivation following membrane repolarization occurs in a monoexponential fashion. Pharmacological depression is obtained by intracellular cesium, tetraethylammonium (TEA), or 4-aminopyridine (4-AP), or external TEA, 4-AP, or barium. In support of tissue culture studies, mature astrocytes obtained from enzymatic isolation techniques (Barres et al., 1990b; Tse et al., 1992) or investigated *in situ* (Steinhäuser et al., 1992; Sontheimer and Waxman, 1993) expressed macroscopic I_{DR} channels with similar kinetic and pharmacological properties.

Analysis of single K^+ channels in astrocytes has demonstrated remarkable diversity with variations in voltage-dependence, conductance, and pharmacological sensitivity (Figure 11-3) (e.g., 201 pS: Sonnhof and Schachner, 1986; 7 and 20 pS: Nowack et al., 1987; 20 and 100 pS: Barres et al., 1988; 45, 67, and 90 pS: Jalonen and Holopainen, 1989). These studies have not, however, demonstrated a single candidate for the channel underlying I_{DR}. At present, no individual channel corresponds to the well-characterized macroscopic current described above. However, it is possible that a variety of channel isoforms, each constructed of different subunits with diverse properties, contribute to the macroscopic voltage- and time-dependent currents.

The I_{DR} may be present throughout cell lineage, from initial precursor to mature astrocyte (Figure 11-4). Expression of the I_{DR} current was observed in a bipotent progenitor cell, termed O-2A, which differentiates into type 2 astrocytes and oligodendrocytes (Raff et al., 1983; Sontheimer et al., 1989; Barres et al., 1990a) and a multipotent EGF-responsive stem cell which differentiates into neurons, astrocytes, and oligodendrocytes (Fraser et al., 1992; Reynolds and Weiss, 1992; Weiss et al., 1992). The current is expressed into maturity (Steinhäuser et al., 1992; Tse et al., 1992; Sontheimer and Waxman, 1993); however, the current density may be reduced during development in a subpopulation of astrocytes (Sontheimer et al., 1992).

Transient A-Type Potassium Channel

The A-type K^+ current (I_A), first described in gastropod neuronal soma (Connors and Stevens, 1971), has been demonstrated in numerous excitable cell types where it modulates action potential frequency (Hille, 1992). The first description of I_A in a glial cell was obtained in type 2 astrocytes derived from optic nerve (Bevan and Raff, 1985; Bevan et al., 1987; Barres et al., 1988; Barres et al., 1990a) (Figure 11-2). The I_A, however, was not expressed in type 1 astrocytes cultured from the optic nerve (Bevan and Raff, 1985; Barres et al., 1990b). Subsequent reports revealed expression of I_A in astrocytes obtained from the cerebral cortex of rat (Bevan et al., 1985) and mouse (Nowack et al., 1987), salamander retina (Newman, 1985), and rat spinal cord (Sontheimer et al., 1992). As described in other cell types, the current activates and inactivates rapidly with depolarization. Neurons typically require a transient hyperpolarization to remove steady-state inactivation. However, the resting membrane potential of glial cells is so negative (-90 mV in 3 mM external K^+) that it is likely there is little steady-state inactivation of I_A at rest. The activation threshold is close to -40 mV and the conductance increase occurs in a sigmoidal fashion, much like the K_{DR}. The time-to-peak is voltage-dependent and with maintained depolarization the current undergoes voltage-dependent

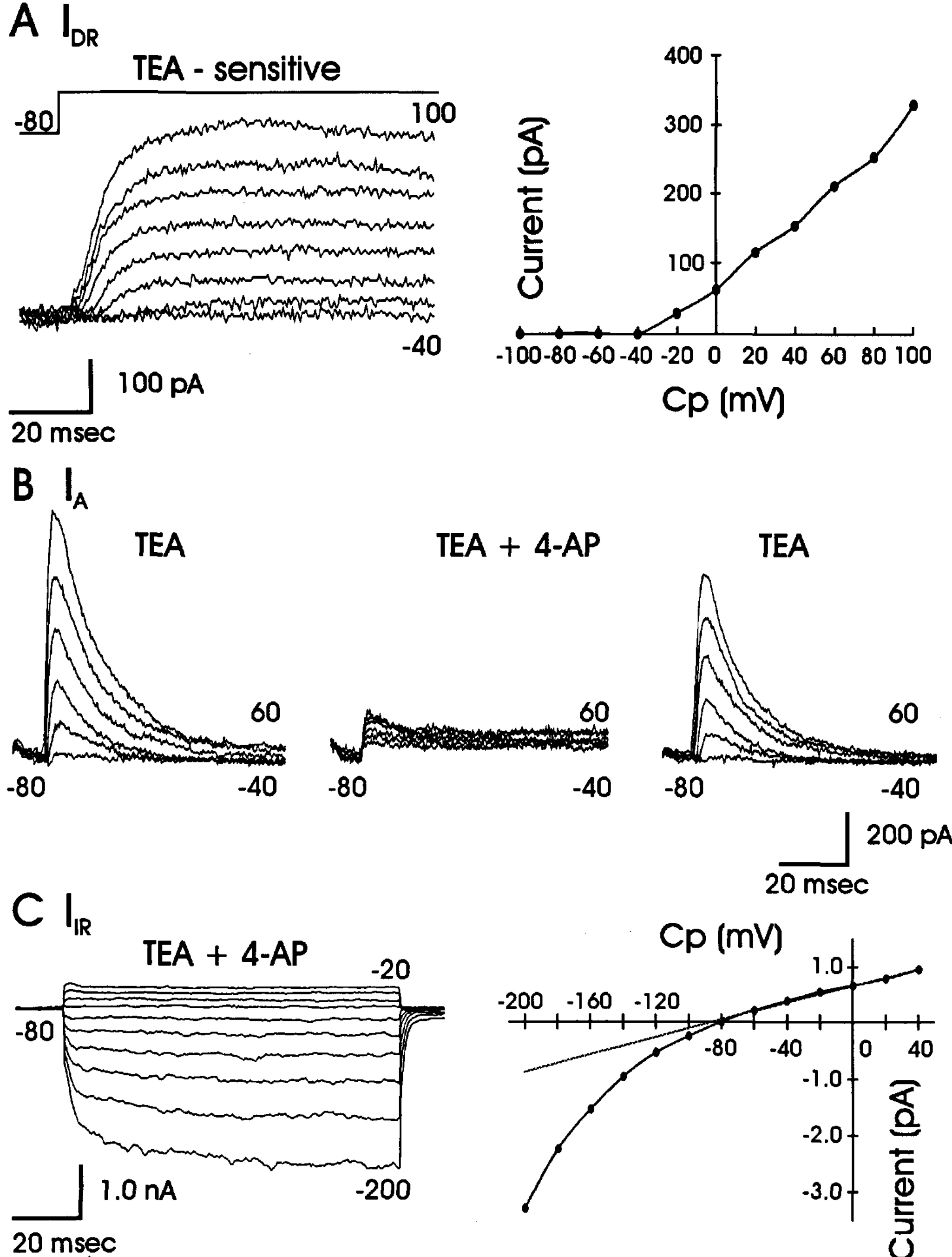

FIG. 11-2. Whole-cell voltage-clamp recordings from acutely isolated hippocampal astrocytes demonstrating the presence of I_{DR}, I_A, and I_{IR}. (A) Membrane potential was stepped from −40 mV to 100 mV from a holding potential of −80 mV in external solutions with or without tetraethylammonium (TEA, 40 mM). The outward current that was blocked by TEA (I_{DR}) is illustrated in the *left panel* and the *I-V* relationship of the peak outward current is plotted. There was no significant inactivation of the current over this time frame. The current had a threshold for turn-on of −40 mV. (B) In the presence of external TEA to block I_{DR} a rapidly inactivating outward current (I_A) was still observed. I_A turned on with a much faster time course than I_{DR} and inactivated almost completely over a time-course in which I_{DR} exhibited no decrement. I_A was totally blocked by external 4-aminopyridine (4-AP) in a reversible manner. (C) When I_{DR} and I_A were both blocked by external TEA and 4-AP an inward current (I_{DR}) was still evoked with hyperpolarizing voltage command pulses. I_{IR} did not exhibit appreciable inactivation at these potentials and turned on at potentials more negative than −80 mV.

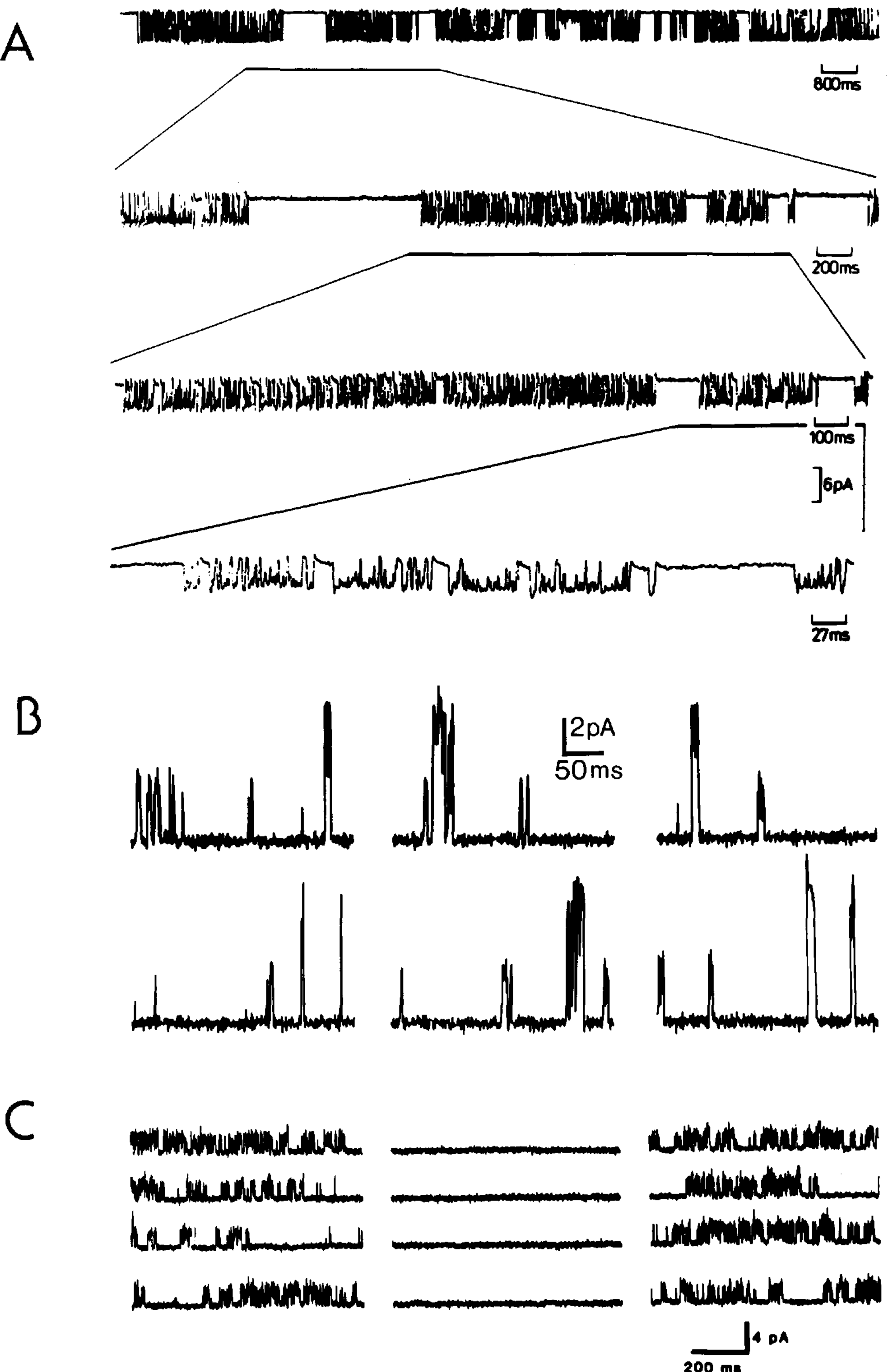

FIG. 11-3. Single-channel recordings from astrocytes illustrating voltage- and calcium-dependent K^+ channels. (A) Single K channel openings exhibited prolonged bursts in an inside-out patch recorded at −40 mV. Burst duration dramatically increased with depolarization. [From Sonnhof and Schachner (1986), with permission.] (B) Recordings from outside-out patches in another study revealed outward currents that appeared to constitute two classes. Mean current amplitudes were 1.6 and 3.7 pA. [From Nowak et al. (1987), with permission.] (C) Calcium-activated K^+ channels recorded from a type 1 cortical astrocyte in cell culture. This channel exhibited voltage and calcium sensitivity and was reversibly blocked by tetraethylammonium (second panel). [From Quandt and MacVicar (1986), with permission.]

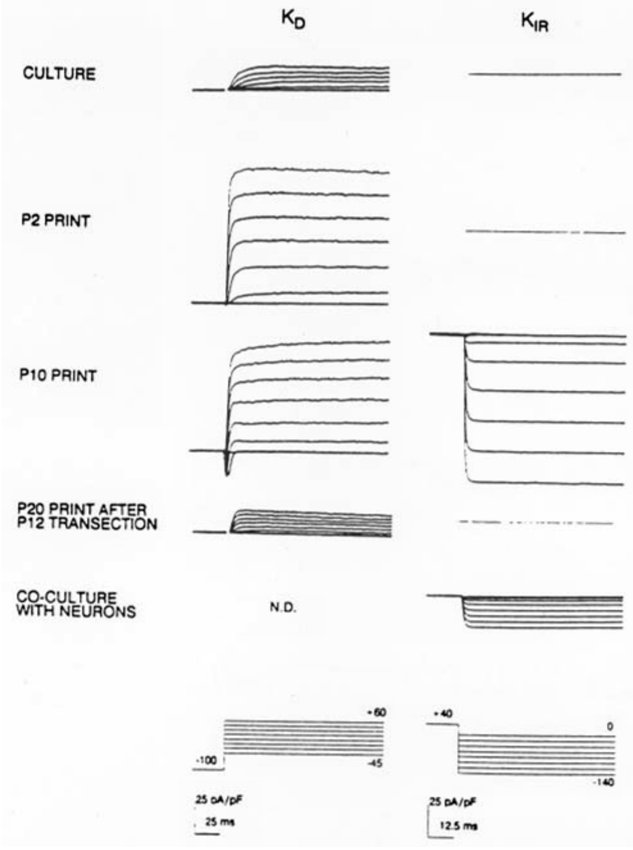

FIG. 11-4. Variability of the expression of I_{DR} and I_{IR} in type 1 astrocytes during development and in different culture conditions. Whole-cell voltage-clamp recordings are illustrated from astrocytes either in culture or prints obtained on postnatal day 2 (*P2*) or day 10 (*P10*). Currents are also illustrated from cells printed on *P20* after optic nerve transection on *P12* and from astrocytes cocultured with neurons for 36 hours. The expression of both I_{DR} and I_{IR} were enhanced during development and were altered by coculture with neurons. [From Barres et al. (1990b), with permission.]

inactivation with a monoexponential time course. I_A is blocked by intracellular cesium or 4-aminopyridine or external 4-AP (at lower concentrations than I_{DR}) and is relatively insensitive to external tetraethylammonium. Since the activation thresholds of the I_{DR} and the I_A currents are similar, current separation and isolation requires subtraction procedures using voltage paradigms (Barres et al., 1988; Sontheimer et al., 1992; Tse et al., 1992; Sontheimer and Waxman, 1993) or pharmacological manipulations (Nowack et al., 1987; Sontheimer et al., 1992; Tse et al., 1992). In support of the tissue culture studies mentioned above, mature type 1 astrocytes obtained from the optic nerve using enzymatic isolation techniques did not express I_A channels (Barres et al., 1990b). However, mature hippocampal astrocytes obtained from enzymatic isolation techniques (Tse et al., 1992) or investigated *in situ* (Steinhäuser et al., 1992; Sontheimer and Waxman, 1993) expressed macroscopic I_A channels.

As with the I_{DR}, single-channel analysis has not yet identified a microscopic candidate that could underlie the macroscopic I_A current (Sonnhof and Schachner, 1986; Jalonen and Holopainen, 1989). Inactivation of I_A is thought to result from movement of a peptide ball that occludes the intracellular side of the channel pore via electrostatic and hydrophobic interactions (Hoshi et al., 1990; Zagotta et al., 1990). Since excised patches were used to identify single astrocytic K^+ channels, it is possible that the inactivation gate was disrupted or, alternatively, the inactivation particle in astrocytes may be a cytoplasmic constituent.

As with the delayed rectifier, the I_A currents may be present throughout cell lineage. Expression of the I_A was observed in bipotent progenitor cells (Raff et al., 1983; Sontheimer et al., 1989; Barres et al., 1990a) and multipotent EGF-responsive stem cells (Fraser et al., 1992; Reynolds and Weiss, 1992; Weiss et al., 1992), which may differentiate into astrocytes. The I_A was also expressed in type 2 astrocytes derived from optic nerve (Bevan and Raff, 1985; Barres et al., 1988) or from spinal cord (Sontheimer et al., 1992). The current is expressed into maturity (Steinhäuser et al., 1992; Tse et al., 1992; Sontheimer and Waxman, 1993); however, the current density may be reduced throughout development in a subpopulation of astrocytes (Sontheimer et al., 1992). The I_A may be developmentally regulated, since the activation and inactivation kinetics of mature astrocytes are at more positive potentials than precursors. For example, bipotent progenitor cells and multipotent EGF-responsive stem cells have inactivation midpoints at -95 mV (Sontheimer et al., 1989) and -74 mV (Fraser et al., 1992a), respectively, while enzymatically isolated astrocytes from mature hippocampus have inactivation midpoints of -60 mV (Tse et al., 1992). It should be noted that space-clamp error may be more pronounced in voltage-clamp studies of astrocytes acutely isolated or in slice preparations. This could contribute to the observed differences in activation and inactivation potentials.

Calcium-Activated Potassium Channel

Two major subtypes of Ca^{2+}-activated K^+ channels ($I_{K(Ca)}$) have been identified based on single-channel conductance, Ca^{2+} sensitivity, voltage-dependence, and pharmacological blockade (Latorre et al., 1989). The maxi $I_{K(Ca)}$ channel (or BK) is characterized by a large conductance (130 to 300 pS) and blockade

by extracellular tetraethylammonium, charybdotoxin, or iberiotoxin, whereas the mini $I_{K(Ca)}$ channel (or SK) is characterized by a smaller conductance (<80 pS) and blockade by TEA or apamin. The presence of $I_{K(Ca)}$ channels on astrocytes was speculated following the demonstration of voltage-activated Ca^{2+} channels (MacVicar, 1984). $I_{K(Ca)}$ currents were first demonstrated in glial cells using whole-cell voltage-clamp recordings of dissociated retinal Müller cells (Newman, 1985). The outward current was activated by depolarization and could be depressed by external TEA or blockade of voltage-dependent Ca^{2+} influx. Single-channel recordings from rat (Quandt and MacVicar, 1986) (Figure 11-3) or mouse (Nowack et al., 1987) cerebral astrocytes have revealed two channel subtypes with properties corresponding to SK (25 pS) and BK (230 pS). Both channels exhibited Ca^{2+} and voltage sensitivity and depression by extracellular TEA.

Expression of the BK current was observed in both bipotent progenitor cells (Raff et al., 1983; Barres et al., 1990a) and mature type 2 astrocytes (Barres et al., 1988), indicating that it may be present throughout cell lineage. The $I_{K(Ca)}$ in both cell types was composed of sustained and transient components and was sensitive to external charybdotoxin or changes in intracellular Ca^{2+} buffering. A BK current was also described in multipotent EGF-responsive stem cells in experiments using the perforated-patch technique to inhibit intracellular dialysis (Fraser et al., 1992; Reynolds and Weiss, 1992; Weiss et al., 1992). Under these conditions, the current displayed weak voltage-dependent inactivation and was sensitive to external iberiotoxin or TEA. Mature type 1 astrocytes obtained from enzymatic dissociation revealed K^+ currents that were sensitive to intracellular Ca^{2+} buffering; however, the K^+ currents were not depressed by external charybdotoxin (Barres et al., 1990b). Thus, the $I_{K(Ca)}$ in this noncultured cell type may be of a type other than the BK channels. Mature astrocytes in kainate-lesioned hippocampal slices appeared to express the SK channel, since a portion of K^+ permeability was sensitive to apamin (Burnard et al., 1990).

Inward Rectifier Potassium Channel

The inward rectifier K^+ channel (I_{IR}), first described by Katz (1949; see Hille, 1992) in muscle cells, is a conductance that increases with hyperpolarization. The first evidence for a glial inward rectifier was obtained using whole-cell voltage-clamp recordings of acutely dissociated retinal Müller cells (Newman, 1985). It was soon clear that the I_{IR} was widely distributed in cultured cells; expression was reported in type 1 (Barres et al., 1990a) and type 2 (Barres et al., 1990b) astrocytes from optic nerve, type 2 astrocytes from rat cortex (Sontheimer et al., 1989), astrocytes from mouse cerebral cortex (Walz and Hinks, 1987), and astrocytes from rat spinal cord (Sontheimer et al., 1992). Expression was detected in astrocytes obtained by enzymatic isolation (Barres et al., 1990b; Tse et al., 1992) (Figure 11-2) or recorded *in situ* (Steinhäuser et al., 1992; Sontheimer and Waxman, 1993). Several properties of the I_{IR} channel are unique and well-conserved, including voltage-dependence of channel activation and conductance, dependence on $[K^+]_0$ and sensitivity to external cesium (Barres et al., 1990a, 1990b) or barium (Sontheimer et al., 1989; Steinhäuser et al., 1992; Tse et al., 1992). However, there appears to be heterogeneity in regard to voltage-dependent inactivation. For example, in a subset of astrocytes, I_{IR} undergoes voltage-dependent inactivation at hyperpolarizing potentials (Steinhäuser et al., 1992; Sontheimer and Waxman, 1993), whereas in other populations the current is noninactivating (Barres et al., 1990a, 1990b; Sontheimer et al., 1992; Tse et al., 1992). The I_{IR} probably plays a dominant role in setting the astroglial resting membrane potential. This is the only K^+ channel type described so far with a large open probability near the equilibrium potential for K^+. Also external Cs^+ or Ba^{2+} depolarize glial cells most likely by blocking I_{IR}. At the microscopic level, single-channel records obtained from acutely isolated retinal Müller cells have revealed heterogeneity of I_{IR} channels with different conductances ranging from 40 to 105 pS in symmetrical K^+ (Brew et al., 1986; Nilius and Reichenbach, 1988).

The I_{IR} channels may be present throughout cell lineage in at least one astrocytic subtype from optic nerve. Expression of I_{IR} was observed in bipotent progenitor cells (Barres et al., 1990a), and current expression was retained in mature type 2 astrocytes (Barres et al., 1988; 1990b). However, the current was not expressed in bipotent progenitor cells obtained from rat brain (Sontheimer et al., 1989) or multipotent EGF-responsive stem cells from mouse striatum (Fraser et al., 1992; Reynolds and Weiss, 1992). The macroscopic current was expressed later in development in both type 1 astrocytes enzymatically obtained from optic nerve (Barres et al., 1990b) and astrocytes cultured from rat spinal cord (Sontheimer et al., 1992). The currents were observed in mature hippocampal astrocytes obtained by enzymatic isolation (Tse et al., 1992) or recorded *in situ* (Steinhäuser et al., 1992; Sontheimer and Waxman, 1993).

The Leak Current

Initial electrophysiological studies on astrocytes using conventional microelectrode techniques revealed a linear current-voltage relation, which reversed close to the resting membrane potential (reviewed by Walz, 1989). Since the resting membrane potential corresponds to the K^+ equilibrium potential, it was assumed that the large K^+ permeability was due to a nonrectifying leak current. Recently, new electrophysiological techniques have been developed, which suggest that, as in neurons, the true electrotonic behavior of glial cells may be masked by conventional intracellular recording. These methods include whole-cell and perforated patch techniques (Hamill et al., 1981; Horn and Marty, 1988). Neurons have much higher values for input resistance when recorded using whole-cell patch-clamp methods, as compared to intracellular recordings using sharp microelectrodes. Comparisons suggest that sharp microelectrodes induce a leak conductance three times greater than that measured by whole-cell patch-clamping (Staley et al., 1992). This leak conductance is generated by membrane damage surrounding the impalement and an increase K^+ permeability evoked by cytosolic elevations in sodium and calcium. The perforated patch method, which does not allow intracellular dialysis, has further confirmed this point (Spruston and Johnston, 1992). Considering the relatively small diameter of an astrocytic soma, one would expect even a greater leak conductance induced by impalement. At present, no quantitative study of the electrotonic structure of astrocytes has been performed in a slice preparation using these techniques. However, these methods may reveal a surprisingly large input resistance, not previously assumed. The current-voltage relation of astrocytes *in vivo* may be far from linear, considering the wide range of voltage-gated channels expressed (Sontheimer, 1992; Sontheimer and Waxman, 1993). Finally, if the electrotonic structure of the astroglial syncytium has been misinterpreted, astrocytes may play a greater role in K^+ siphoning than previously believed.

Spatial Distribution of Channels

It has been shown in retinal Müller cells that particular regions of glial membranes may contain higher densities of K^+ channels or K^+ channels with a greater conductance (see Chapter 47, this volume). Experiments using focal K^+ ejection have demonstrated that the magnitude of the endfoot responses are exceptionally large, indicating a greater K^+ conductance level (Newman, 1984). In support of these studies single channel analysis has demonstrated that I_{IR} channels are distributed unevenly across the membrane. The vitreal process and endfoot contain a greater density of I_{IR} channels (Brew et al., 1986). In fact, removal of the endfoot results in a dramatic increase in cellular resistance (reviewed by Newman, 1987). These studies indicate that 95% of the total conductance lies in or around the endfoot removed by a microdissection procedure. In mammalian retinal glial cells, a second nonrectifying K^+ channel was found, with a much greater single-channel conductance to facilitate K^+ efflux (Nilius and Reichenbach, 1988). This precise segregation of K^+ influx channels (I_{IR}) and efflux channels may allow astrocytes to more efficiently clear $[K^+]_0$ from areas of accumulation around active neurons (spatial buffering) or augment the activity of K^+ transporters (Walz, 1989).

Other Relevant Currents

To maintain electroneutrality, the movement of K^+ across biological membranes is often accompanied or restricted by movements of Cl^- (Boyle and Conway, 1941). In astrocytes, the Cl^- equilibrium potential is positive to the resting membrane potential. In fact, the equilibrium potential may be as positive as -35 mV due to the activity of two inwardly directed transporters, a Na^+-K^+-$2Cl^-$ cotransport and a Cl^--HCO^-_3 exchange (Kettenmann, 1990; Kimelberg, 1990). The first demonstration of a Cl^- current in astrocytes was obtained via whole-cell voltage-clamp recordings of macroscopic currents (Bevan et al., 1985). Ionic identification was based on analysis of reversal potentials and ion substitution experiments. Subsequent studies were unable to identify a macroscopic Cl^- current, although single channels were identified in excised patches at the microscopic level (Barres et al., 1988; 1990b). The discrepancy between the lack of observable macroscopic currents and the presence of single channel currents in excised patches suggest that channel activity is regulated by cytoplasmic factors. Further evidence for this phenomenon is inferred by the lack of single-channel recordings in the cell-attached recording mode and their ensuing appearance several minutes following excision (Sonnhof, 1987). Nonetheless, excised recordings have revealed two channel subtypes based on conductance levels. The large conductance channels are 260 (Barres et al., 1988), 385 (Nowack et al., 1987) or 465 pS (Sonnhof, 1987) and open in response to depolarization. The activation threshold is close to -40 mV, much like I_{DR} and I_A. The small conductance channels are 5

(Nowack et al., 1987), 25 or 60 pS (Barres et al., 1990b), and at least one subtype of the small conductance channels open with hyperpolarization (Nowack et al., 1987). A Cl^- flux may also be initiated by opening ligand-gated channels. Astrocytes in culture (Bormann and Kettenmann, 1988) or isolated following enzymatic treatment (Fraser et al., 1995) express $GABA_A$ receptors which mediate a large Cl^- conductance.

Elevations in cytosolic Ca^{2+}, from influx through either voltage- or ligand-gated channels, can regulate K^+ flux across the cell membrane (Latorre et al., 1989). The first report of Ca^{2+} channels in astrocytes came from current-clamp studies (MacVicar, 1984). Subsequent reports, using whole-cell voltage-clamp techniques, identified both low-voltage activated (Barres et al., 1988; 1990b; Puro and Mano, 1991) and high-voltage activated Ca^{2+} channels (Newman, 1985; Barres et al., 1988; 1990b; MacVicar and Tse, 1988; Corvalan et al., 1990; Puro and Mano, 1991). Most of these studies were on cultured astrocytes exposed to dBcAMP (MacVicar, 1984) or serum (Barres et al., 1989); however, a high-threshold Ca^{2+} influx was recently revealed in enzymatically isolated hippocampal astrocytes (Duffy and MacVicar, 1994). An elevation in intracellular Ca^{2+} may also occur by activation of ligand-gated receptors. An obvious transmitter candidate is glutamate. Indeed, cultured hippocampal astrocytes (Glaum et al., 1990; Cornell-Bell et al., 1990) or Bergmann glial cells studied in either culture (Burnashev et al., 1992) or *in situ* (Müller et al., 1992) express Ca^{2+}-permeable AMPA-kainate receptors.

OLIGODENDROCYTES AND SCHWANN CELLS

While many functions have been attributed to glial cells, a functional role is most clearly defined for myelinating peripheral nervous system Schwann cells and central nervous system oligodendrocytes. Myelination restricts Na^+ influx to short axon segments (nodes of Ranvier), thereby greatly increasing action potential velocity and reducing energy consumption for ion redistribution (Hille, 1992). Several observations, however, indicate that these cells have other functions. First, many Schwann cells and oligodendrocytes do not produce myelin, but rather extend processes which interdigitate between many small diameter axons. Second, both Schwann cells (Evan et al., 1985, 1992; Jahromi et al., 1992) and oligodendrocytes (Vartanian et al., 1988; Butt and Tutton, 1992) express neurotransmitter receptors, which may allow chemical communication with axons. Third, these cells express voltage-gated ionic channels, the most prominent of which are several subtypes of K^+ channels. These channels may confer several important functional attributes to oligodendrocytes and Schwann cells, including spatial buffering of interstitial K^+ ions and the modulation of cell proliferation during early development and following nerve degeneration.

Potassium Channel Expression and Modulation in Oligodendrocytes

As in astrocytes, the membranes of oligodendrocytes are predominately permeable to K^+. In oligodendrocytes from explant cultures of mouse spinal cord, Kettenmann et al. (1983b) observed that E_m changed 61 mV per tenfold change in $[K^+]_0/[K^+]_i$, consistent with the Nernst relation for an exclusively K^+-permeable membrane. Similarly, the E_m-$[K^+]_0$ relation in oligodendrocytes from the intact optic nerve was consistent with a K^+-selective membrane (Butt and Tutton, 1992), suggesting that oligodendrocyte *in situ* have similar biophysical properties. Methods for culturing relatively pure oligodendrocyte populations (McCarthy and De Vellis, 1980) and patch-clamping at both the whole-cell and single channel levels (Hamill et al., 1981) have greatly facilitated the description of the K^+ currents and channels which underlie this large membrane K^+ permeability. These studies have revealed that cells of the oligodendrocyte lineage express most of the K^+ channel subtypes that have been described in neurons and astrocytes, including I_{IR}, I_{DR}, I_A, and $I_{K(Ca)}$.

The specific complement of K^+ channels expressed by oligodendrocytes is highly variable, depending on species, culture methods, and developmental stage. For example, cultured ovine oligodendrocytes (Soliven et al., 1988a, 1988b), mouse oligodendrocytes (Sontheimer and Kettenmann, 1988) and serum-free cultures from rat optic nerves (Barres et al., 1990a) expressed all three K^+ currents simultaneously. Rat optic nerve cultures containing serum expressed either I_{DR} and I_{IR} (Barres et al., 1990b) or only I_{IR} (Barres et al., 1988). On the other hand, oligodendrocytes cultured from mouse brain (Sontheimer et al., 1989b) and cultured adult bovine oligodendrocytes (McLarnon and Kim, 1989b) were found to express only I_{IR}. Likewise, oligodendrocytes cultured from adult humans expressed a prominent I_{IR}, while outward channel density was quite low (McLarnon and Kim, 1989a). Oligodendrocytes cultured from fish optic nerve, however, express both outward K^+ currents but not I_{IR} (Hoppe et al., 1991; Glassmeier et al., 1992). A completely passive *I-V* relation, indicating no (measurable) voltage-dependent ionic

channels, was reported in cultured oligodendrocytes from rat optic nerve (Bevan and Raff, 1985). There is also considerable variability in oligodendrocyte ionic channel phenotype within a given culture (Sontheimer and Kettenmann, 1988).

Oligodendrocytes *in situ* also express voltage-gated K^+ currents (Barres et al., 1990a; Berger et al., 1991), and again, ionic channel expression pattern is heterogeneous. Barres et al. (1990a) found that oligodendrocytes, acutely isolated from postnatal day 7 rat optic nerve, expressed all three current components. Conversely, Berger et al. (1991) found mainly I_{DR} in oligodendrocytes from slices of postnatal days 6 to 8 mouse corpus callosum, while in older animals (postnatal days 10 to 13), no voltage-gated currents were reported.

The influence of developmental plasticity has been thoroughly investigated in cultures of developing oligodendrocyte precursors (Soliven et al., 1988b; Sontheimer et al., 1989; Barres et al., 1990a). By correlating ionic channel expression pattern with the development changes in surface antigen expression, these studies have found that channel phenotype becomes less complex as oligodendrocyte differentiation progressed. In one such study, the A2B5 immunopositive oligodendrocyte-type 2 astrocyte precursor cell (O-2A cell) (Raff et al., 1983) expressed I_{DR} and I_A, as well as a $[Ca^{2+}]_i$-dependent K^+ current ($I_{K(Ca)}$) and I_{Na} (Sontheimer et al., 1989). After commitment to the oligodendroglia lineage, however, all these currents were downregulated and I_{IR} density increased. In a similar study, I_{DR} and I_{IR} remained in mature cells (Barres et al., 1990a). Changes in channel expression were also observed in aging oligodendrocyte cultures (Soliven et al., 1988b); outward currents were downregulated slightly, while I_{IR} increased significantly over the first 10 days after plating.

Potassium Current Kinetics and Single-Channel Properties in Oligodendrocytes

At the level of the macroscopic current, I_{DR} and I_A activate at potentials positive of −50 to −40 mV (Barres et al., 1990; Sontheimer and Kettenmann 1988; Soliven et al., 1988a, 1988b; Hoppe et al., 1991; Glassmeier et al., 1992). Like the neuronal I_A, oligodendrocyte I_A shows voltage-dependent inactivation (inactivation rate increasing with depolarization) and sensitivity to external 4-aminopyridine (Soliven et al., 1988a, 1988b; Sontheimer et al., 1989; Glassmeier et al., 1992). I_A is largely inactivated at resting E_m. In one study (Soliven et al., 1988b) I_A showed approximately 80% inactivation at −60 mV, suggesting that activation only occurs with depolarization from a relatively hyperpolarized E_m. Conversely, I_{DR} shows little voltage-dependent inactivation and is more sensitive to external TEA. I_{IR} is activated by hyperpolarization below −70 to −80 mV, and current magnitude increases with elevations in $[K^+]_0$. I_{IR} is, in most instances, noninactivating or slowly inactivating. However, at very negative potentials, inactivation of I_{IR} has been shown (Barres et al., 1988).

The first measurement of single-channel currents in oligodendrocyte revealed that these cells expressed a prominent voltage-independent, high-conductance (70 pS) K^+ channel, which may account for the large resting K^+ conductances (gk) of oligodendrocyte membranes (Kettenmann et al., 1982). An unequivocal link between many channel subtypes measured at the single-channel level and identified macroscopic currents has only been firmly established for I_{IR}. With depolarization, I_{IR} open channel probability and mean channel open time increase. However, conductance is minimal. Hyperpolarizing the membrane to values more negative than E_K results in short high-conductance openings, and conductance increases with $[K^+]_0$. Single-channel behavior is similar across mammalian oligodendrocytes. In cultured adult human oligodendrocytes, single-channel conductance was 29 pS in 140 mM $[K^+]_0$ (McLarnon and Kim, 1989b). Nearly identical channel activity (30 pS in 130 mM K^+) was measured in cultured rat oligodendrocytes from optic nerve (Barres et al., 1988) and in cultured ovine cells (28 pS in 140 mM K^+) (Soliven et al., 1989). In addition, rat oligodendrocytes also expressed a higher conductance I_{IR} channel of 120 pS.

Functions of Oligodendroglial Potassium Channels

The activation range of macroscopic I_{DR} and I_A implies that these conductances would be most active during very large depolarizations, possibly in response to neurotransmitter stimulation (Barres et al., 1990a, 1990b; Butt and Tutton 1992) or in immature cells which express Na channels (Barres et al., 1990a, 1990b; Sontheimer et al., 1989). The biophysical properties of I_{IR} suggest, more clearly, several possible functional roles in oligodendrocyte physiology. I_{IR} channels are most active near the resting E_m, implying that I_{IR} must play a dominant role in setting resting E_m. Also, because K^+ influx increases as the difference between E_m and E_K increases, I_{IR} could, in manner analogous to that proposed for astrocytes, allow oligodendrocytes to spatially buffer the accumulation of K^+ ions resulting from action potential repolarization. In fact,

white matter $[K^+]_0$ may increase substantially during nerve excitation, necessitating strong K^+ buffering capacity. In corpus callosum oligodendrocytes, the reversal potentials of tail currents following depolarizating pulses became more positive as pulse duration was increased, indicative of a depolarizing shift in E_K concomitant with interstitial K^+ accumulation (Berger et al., 1991). The actual role of oligodendrocytes in spatial buffering, however, is still uncertain. Such a process would be greatly facilitated by spatial segregation of rectifying and nonrectifying ion channels (i.e., K^+ influx and efflux pathways), but unlike Müller cells of the retina (Brew et al., 1986; Newman, 1986; Nilius and Reichenbach, 1988) and mammalian Schwann cells (Wilson and Chiu, 1990b) channel segregation has not been demonstrated in oligodendrocytes. Moreover, spatial buffering mediated by K^+ channels (as opposed to K^+ transporters) depends on electrotonic coupling between cells to form an electrotonic syncytium. While oligodendrocytes were electrotonically coupled to neighboring oligodendrocytes (Kettenmann et al., 1983a; Kettenmann and Ransom, 1988) and astrocytes in coculture (Ransom and Kettenmann, 1990), the coupling ratio was much smaller than that between astrocytes. Thus, it has been suggested that white matter astrocytes are more important than oligodendrocytes in buffering K^+. On the other hand, cultured rat oligodendrocytes did express a very large conductance I_{IR} channel (Barres et al., 1988), and it is this channel which has been postulated to be of special importance for K^+ buffering in mammalian retina (Nilius and Reichenbach, 1988).

Oligodendrocytes may also actively accumulate K^+ ions by KCl influx. Oligodendrocytes express large conductance anion channels (Barres et al., 1988), which are a prerequisite to this process. Moreover, $[K^+]_0$-dependent $[K^+]_i$ increases have been demonstrated in cultured oligodendrocytes with $[K^+]_i$-sensitive microelectrodes (Kettenmann et al., 1983b) and radioactive tracer flux (Hertz et al., 1990).

K^+ currents may modulate oligodendrocyte differentiation, as maturation is accompanied by stereotypic changes in ionic channel expression patterns (Soliven et al., 1989; Sontheimer et al., 1989; Berger et al., 1991) and channel modulation can influence differentiation (Soliven et al., 1988a, 1989). In cultured ovine oligodendrocytes, transient and sustained outward K^+ currents developed within 1 to 2 days after plating, while I_{IR} did not appear until several days later (Soliven et al., 1988a). The outward current was inhibited by analogues of cAMP, the adenylate cyclase activator forskolin, β-adrenergic receptor stimulation (which is coupled to adenylate cyclase), and the protein kinase C activator phorbol esters. *In vitro*, these second messenger systems are also activated by substrate attachment, which in turn leads to myelinogenesis (Vartanian et al., 1986). Thus, there may be a causal link between ionic channel expression and myelinogenesis.

Finally, while variability of ionic channel expression pattern is well described, less clear is the extent to which these differences reflect divergence in cell function. However, an intriguing hint is provided by fish oligodendrocytes. Unlike many oligodendrocytes, these cells do not express I_{IR} (Hoppe et al., 1991; Glassmeier et al., 1992). In this regard, they are similar to mature Schwann cells of higher vertebrates. In addition, these cells share several surface antigens with Schwann cells (Jeserich and Waehneldt, 1987). Significantly, like peripheral Schwann cells and unlike most central nervous system oligodendrocytes, these cells may provide an environment conducive to axon regeneration (Bastmeyer et al., 1991).

Potassium Channel Expression and Modulation in Schwann Cells

Voltage-dependent K^+ channels were first reported on cultured Schwann cells by Chiu et al. (1984). Like oligodendrocytes, Schwann cells express an array of K^+ channel types with expression patterns varying markedly between species. Howe and Ritchie (1988) observed two pharmacologically separable outward K^+ current components in cultured rabbit Schwann cells, one sensitive to both 4-AP and TEA, and one largely resistant to 4-AP but TEA-sensitive. This 4-AP-sensitive current has been further differentiated into two subcomponents, a low-threshold current, sensitive to α-dendrotoxin, and a higher-threshold current that had a lower 4-AP-sensitivity, was insensitive to α-dendrotoxin, and showed slower activation with depolarization (Baker et al., 1993). However, neither 4-AP-sensitive component resembled I_A, the main 4-AP-sensitive I_K in other glia. On the basis of their slower inactivation kinetics and greater TEA-sensitivity, these channels resemble axonal delayed rectifiers (I_{DR}). A 4-AP-sensitive, inactivating (I_A-like) current has, however, been reported in Schwann cells from mouse dorsal root ganglion along with a less 4-AP-sensitive, more TEA-sensitive sustained current (Amedee et al., 1991). Schwann cells also express an inwardly rectifying current (I_{IR}) (Konishi, 1990b, 1992; Wilson and Chiu 1990b), the expression of which is sensitive to the developmental state (see below). The relative density of these currents vary considerably between species.

Konishi (1989) compared outward K^+ currents measured from rabbit, rat, mouse, and human cultured Schwann cells. Transient currents were virtually absent in rabbit but dominated in rat. Mouse and human Schwann cells expressed both components. The I_K in acutely isolated mouse Schwann cells does not have a large inactivating component, however (Konishi, 1990a, 1990b; Wilson and Chiu 1990a), so I_{DR} and I_{IR} are the dominant currents expressed *in situ.*

The ionic channel phenotype of Schwann cells is very sensitive to the developmental state and follows different developmental patterns, depending on whether cells are myelinating or nonmyelinating (Figure 11-5). In one culture study, mature myelinating cells did not express measurable voltage-gated gk, while nonmyelinating cells expressed a prominent I_{DR} (Konishi, 1990a). Similarly, cells acutely isolated from embryonic mouse nerve (before the onset of myelination) expressed I_{DR} (Konishi, 1990a, 1990b). However, in neonatal nerve, whole-cell gk of myelinating cells increased and E_m hyperpolarized. These biophysical changes were due to the expression of a Ba^{2+}-sensitive I_{IR} in addition to I_{DR}. Over the several weeks following birth, both K^+ currents were downregulated in myelinating cells, and E_m became positive to E_K. A similar patter of rapid up- and downregulation of I_{DR} and I_{IR} was observed in myelinating cells from rat sciatic nerve (Wilson and Chiu, 1990a). In nonmyelinating cells, on the other hand, neither current magnitude (of I_{DR}) nor E_m changed significantly with age (Konishi, 1990a, 1990b).

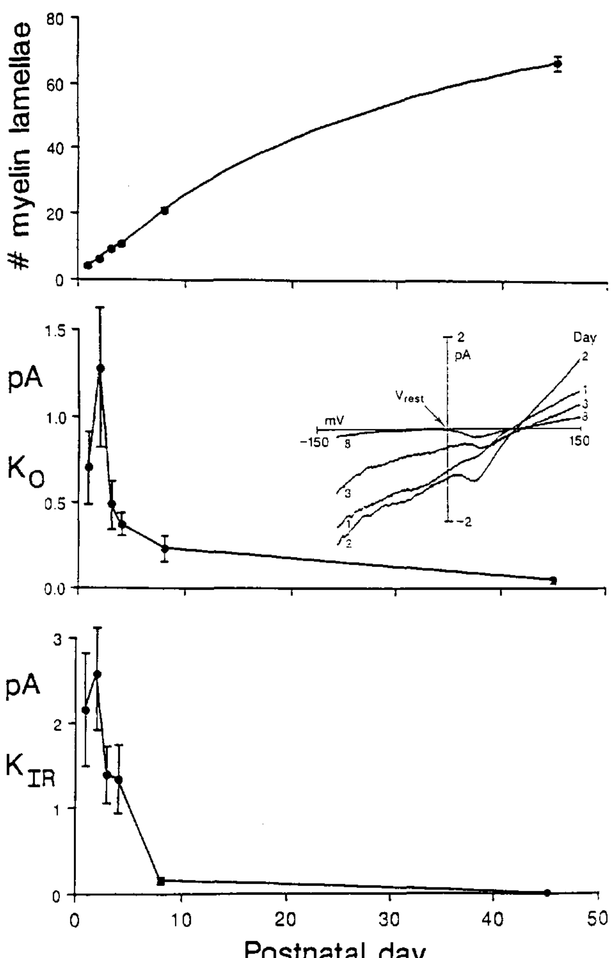

FIG. 11-5. The expression of K^+ currents in Schwann cells changes during development and diminishes during myelination. Current amplitudes were determined from the ramp *I-V* relationship (*insert*). The number of myelin lamellae was determined in electron microscopy by counting the number of major dense lines in cells associated with only one axon. Myelin counts were obtained from nerves contralateral to those used in electrophysiology. [From Wilson and Chiu (1990a), with permission.]

Many aspects of this developmental pattern are recapitulated following nerve transection (Chiu, 1988; Chiu and Wilson, 1989). In Schwann cells surrounding the distal nerve stump, ionic channel expression changed depending whether cells were myelinating or nonmyelinating. In nonmyelinating cells, I_K was slightly reduced and E_m did not change, while in myelinating cells, the TEA-sensitive outward K^+ current was upregulated, and E_m hyperpolarized. Thus, myelinating Schwann cells reverted to a phenotype similar to that observed in neonatal nerve when myelin is first produced.

An important question which arises from these studies is whether the apparent downregulation of ionic channels is due to fewer membrane channels or to migration of channels from the soma (the site of whole-cell voltage-clamp recordings) to less accessible areas of the cell membrane. To address this question, Chiu and Wilson (1990b) made cell-attached patch recordings from adult rat Schwann cells at the paranodal region. Significantly, both I_{DR} and I_{IR} channels were found, indicating that at least some of the apparent downregulation may be due to insertion of channels on electrotonically distant membranes.

The external signals which regulate ionic channel phenotype have also been investigated. Elevations in the intracellular cyclic AMP level is an ubiquitous trigger governing many aspects of Schwann cell differentiation (Sobues et al., 1986). Given the correlation between I_{IR} expression and differentiation it is not surprising that cAMP does have effects on Schwann cell ionic channel expression. In culture, Schwann cells from neonatal mouse lose I_{IR}, while incubation in cell permeant cAMP analogues trigger the reexpression of I_{IR} (Konishi, 1992).

Potassium Current Kinetics and Single-Channel Properties in Schwann Cells

In general, both whole-cell and unitary current properties of these K^+ currents are similar to those observed in other glial and neuronal cells. The channel that underlies the macroscopic I_{IR} of rat sciatic nerve Schwann cells was found to be quite similar to the I_{IR} in other glia, with a single-channel conductance of 35 pS (in 160 mM K^+), and showing significant $[K^+]_0$ sensitivity (Wilson and Chiu 1990a). A slightly lower gk was observed in cultured human Schwann cells (18 pS in 140 mM K^+) (McLarnon and Kim 1991) and rat Schwann cells at the paranode (25 pS) (Wilson and Chiu, 1990b). Moreover, like other I_{IR} channels opening probability increased with depolarization, reaching a maximum around E_m. The 4-AP-sensitive I_{DR} channel has a slope conductance of 12 to 14 pS (Schrager et al., 1985; Wilson and Chiu 1990a, 1990b; Howe et al., 1992), similar to the neuronal I_{DR} channel (Wilson and Chiu, 1990b), while the 4-AP-insensitive outward rectifier has a single channel conductance of around 60 pS (Howe et al., 1992).

Functions of Schwann Cell Potassium Channels

From a functional standpoint, the expression of I_{IR} may indicate that Schwann cells play some role in the buffering of interstitial K^+. The finding of I_{IR} channels opposed to paranodal regions (Wilson and Chiu, 1990b) where interstitial K^+ accumulation may be highest (Chiu, 1991), or during developmental periods where accumulation may be potentiated (Connors et al., 1983) is suggestive of such a role. Also, elevated external $[K^+]_0$ potentiated inward K^+ currents through I_{DR} (Verkhratsky et al., 1991), suggesting that these channels also may contribute to K^+ buffering. Elevations in extracellular pH, which accompany nerve activity under some conditions (Jendelova and Sykova, 1991) also potentiated inward K^+ currents (Hoppe et al., 1989). However, myelinated Schwann cells lack dye coupling (Konishi, 1990b), a possible prerequisite for significant K^+ clearance. It is also possible that Schwann cells could, in a manner analogous to that proposed for astroglial cells or oligodendrocytes, accumulate K^+ by K^+ and Cl^- influx. High-conductance anion channels (gk > 300 pS) have been measured from cultured human (McLarnon and Kim, 1991) and rabbit (Howe and Ritchie, 1988), Schwann cells and from rat spinal root Schwann cells in freshly isolated nerve (Quasthoff et al., 1992). It is clear that I_{IR} does play a dominant role in setting the resting membrane potential, since Schwann cell E_m hyperpolarizes as I_{IR} expression increases (Chiu and Wilson, 1989; Konishi, 1990b). Modulation of E_m may, in fact, be the most important role of I_{IR} channels given that E_m seems to be an important modulator of Schwann cell proliferation. Increases in I_K and hyperpolarizations of E_m following Wallerian degeneration (Chiu and Wilson, 1989) were accompanied by proliferation as indicated by increases in [^{3}H]thymidine incorporation. Significantly, K^+ channel antagonists blocked proliferation with approximately the same potency as they blocked ion channels (Chiu and Wilson, 1989; Konishi, 1989b). However, the mechanism by which low E_m induces proliferation is not clear.

POTASSIUM CHANNELS IN MICROGLIAL CELLS

Microglial cells represent the resident brain population of macrophages (see Perry and Gordon, 1988; Thomas, 1992; Benveniste, 1993; and Chapter 10, this volume for reviews of microglia origins and physiology). The question of their electrophysiological properties is of some interest because the ionic channel expression pattern of immune cells is intimately related to developmental state, and can be altered by mitogens which regulate immune cell function (Lewis and Cahalan, 1988). For example, K^+ channel activity increases during phagocytosis (Ince et al., 1988). In microglial cells cultured from rat brain Kettenmann et al. (1990) observed an inwardly rectifying current with similar single channel conductance to other glial inward rectifiers (30 pS in 150 mM K^+) but no depolarization-activated outward current (Figure 11-6). In contrast both inward and outward K^+ currents were reported in cultured microglia (Korotzer and Cotman, 1992). These authors also noted that ramified microglia were more likely to express I_{Na} than amoeboid cells, suggesting that ionic channel expression may indeed be correlated with physiological state. Again the functional role played by these channels is not clear. As pointed out in Kettenmann et al. (1990) the inward rectifier could, by maintaining the resting potential near E_K, provide an electrochemical driving force for transport. Also, inward rectification would substantially increase membrane input resistance during depolarization. This in turn would greatly amplify the effect of any depolarizing signal from Na channel opening (Korotzer and Cotman, 1992) or neurotransmitter stimulation (Kettenmann et al., 1993).

CONCLUSIONS

The predominant electrophysiological characteristic of glial cells is still the high relative K^+ permeability

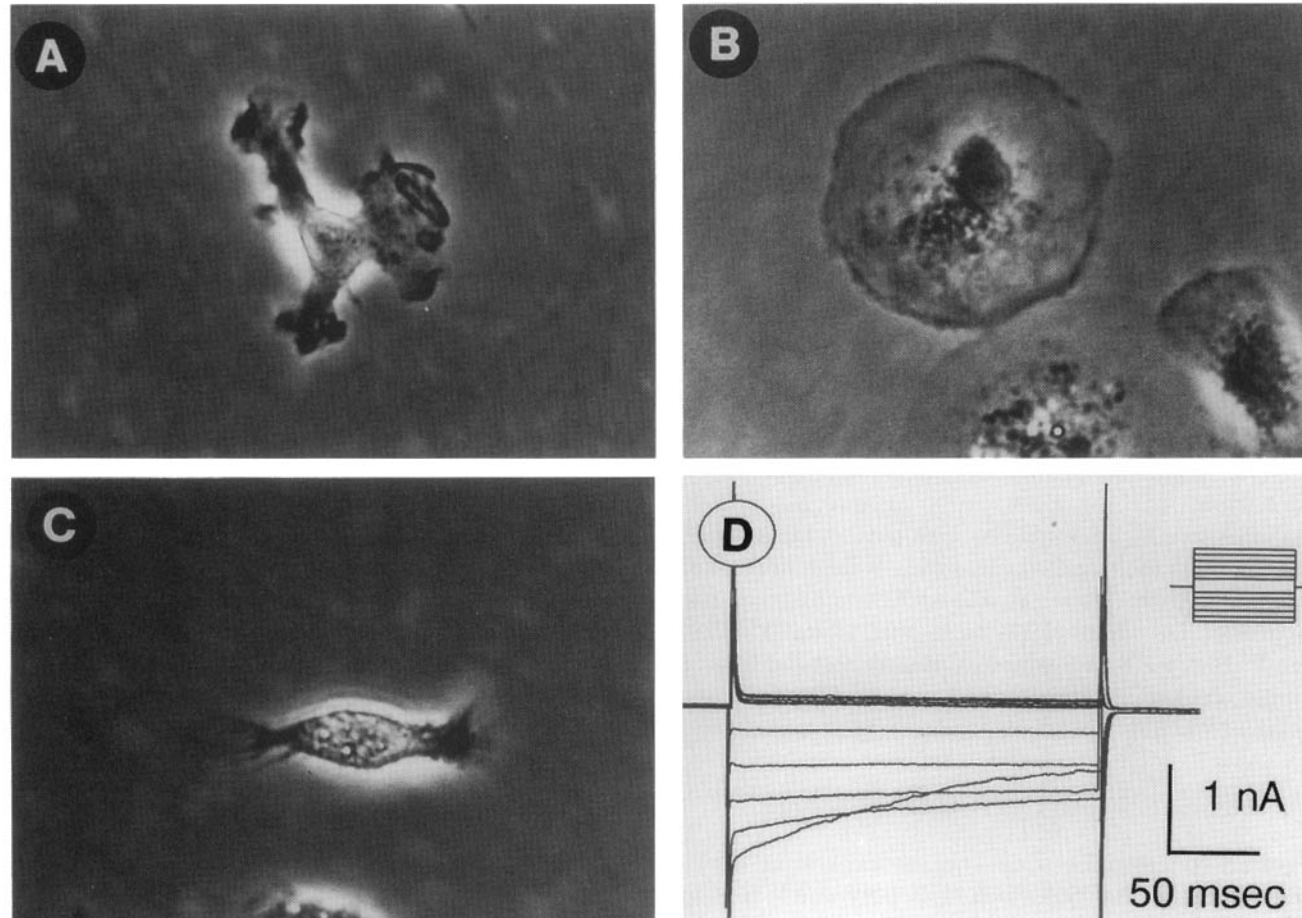

FIG. 11-6. Whole-cell voltage-clamp recordings obtained from microglial cells in cell culture. (A–C) Photomicrographs of microglial cells in a purified culture illustrating their diverse morphologies. Whole-cell voltage-clamp recordings were obtained from the displayed cells. (D) Typical pattern of membrane currents obtained from a microglial cell at a holding potential of −70 mV. Voltage-gated currents (I_{IR}) were observed only when the cell was hyperpolarized during voltage command steps of 20 mV increments. Note that I_{IR} inactivates at more hyperpolarized potentials. [From Kettenmann et al. (1990), with permission.]

of their membrane. The possibility that extracellular potassium is regulated by glial cells (as discussed in Chapter 47, this volume) is based upon our understanding of the membrane properties of these cells. The experimental work of the last few years has revealed that glial cells have many types of K^+ channels. The presence of several species of voltage gated channels suggests a dynamic nature of their K^+ permeability. A major challenge in the near future will be to synthesize the experimental observations of the diverse K^+ channels within a conceptual framework of glial function.

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12 Voltage-gated sodium and calcium channels

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Little was known about the electrophysiological properties of the satellite cells of the nervous system (Schwann cells in the peripheral nervous system; astrocytes and oligodendrocytes in the central nervous system) until the seminal work of Kuffler, Nicholls, and Orkand in 1966 on the glial cells of the optic nerve of the mudpuppy, *Necturus*. They showed not only that the resting potential of these cells (about −90 mV) was determined by the K^+ gradient across the membrane, but also that during impulse activity the K^+ released by axons accumulates in the intracellular clefts, thereby reducing the glial membrane potential. This work clearly established the presence of K^+ channels in the glial cell membrane, and it set the stage for the remarkable period of frenzied discovery that was to occur in the 1980s.

The amphibian K^+ channel described by Kuffler and collaborators was "passive" in the sense that the membrane resistance did not change when the resting potential was displaced by 100 mV or more in either direction. Nor were glial cells found to generate electrical signals when the membrane was depolarized. In other words, none of the then known properties of glial cells suggested the presence of the voltage-gated channels (K^+, Na^+, Ca^{2+}) that are used by the nervous system to generate nerve impulses; and there the position rested for the next 25 years, until the advent of patch-clamp techniques allowed the extension of the earlier work on amphibian glial cells to mammalian glia.

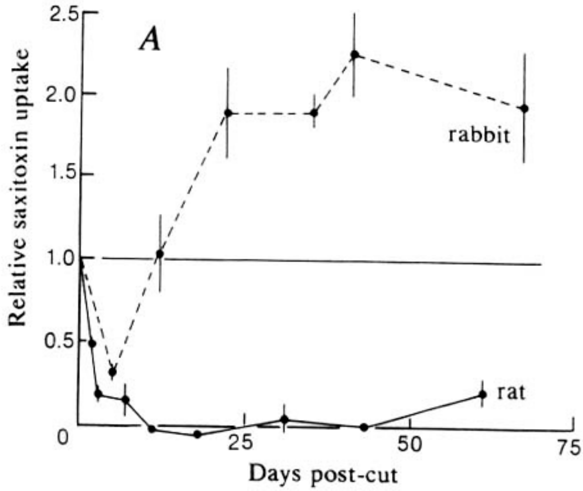
The first report of K^+ channels in mammalian oligodendrocytes appeared in 1982 (Kettenmann et al., 1982), and in 1984 a calcium-activated cation-selective channel was described in rat cultured Schwann cells that was equally permeable to Na^+ and K^+ ions but not detectably permeable to Ca^{2+} ions (Bevan et al., 1984b). Although the opening probability of the latter was somewhat affected by membrane potential neither of these two channels could strictly be called voltage-gated. However, in the decade that followed, it became clear that all the satellite cells of the nervous system (oligodendrocytes, astrocytes, Schwann cells) are liberally endowed with a large repertoire of voltage-gated ion channels. In 1984, voltage-gated Ca^{2+} channels were described in astrocytes by MacVicar (1984); and Hodgkin Huxley type K^+ and Na^+ channels were described for both Schwann cells and astrocytes (Bevan et al., 1984a; Chiu et al., 1984). Voltage-gated anion channels were described for astrocytes in 1985 (Bevan et al., 1985) and for Schwann cells in 1988 (Howe and Ritchie, 1988). The present chapter focuses on the electrophysiological properties of the satellite cell voltage-gated Na^+ channels. Ca^{2+} channels will also be discussed, but briefly because so far they have been less extensively studied.

SCHWANN CELLS

Sodium Channels in Schwann Cells

Saxitoxin Binding. The first suggestion that the satellite cells of the nervous system express neuronal-type Na^+ channels came from experiments where a highly specific marker for Na^+ channels, saxitoxin (STX), was used to follow the disappearance of axons from sectioned rat and rabbit sciatic nerves undergoing Wallerian degeneration (Ritchie and Rang, 1983). In the rat (Figure 12-1A) the uptake of [^{3}H]STX virtually completely disappeared in a few days after section, as would be expected from the subsequent degeneration and loss of axons. Completely unexpected, however, was the finding in sectioned rabbit nerve. After an initial fall the binding began to increase; and a month after nerve section, the now axon-free degenerated distal nerve stump expressed about twice as many STX-binding sites as did the control nerve.

Electrophysiological Experiments. Electrophysiological experiments rapidly showed that the STX binding indeed reflected expression of voltage-gated Na^+ channels in Schwann cells (Chiu et al., 1984; Shrager et al., 1985; Howe and Ritchie, 1990). Figure 12-1B shows a typical whole-cell patch-clamp recording from a rabbit Schwann cell. As can be seen from the corresponding current-voltage plot (Figure 12-1C)



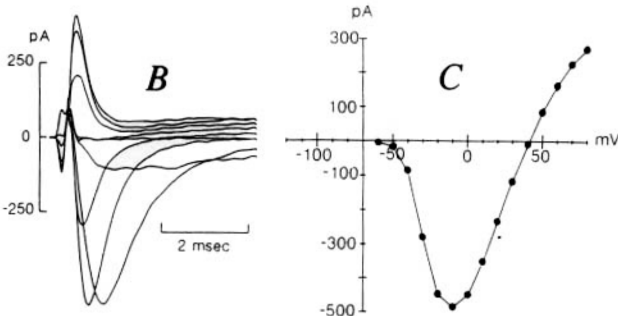


FIG. 12-1. Na^+ channels in Schwann cells. (A) Binding of [^{3}H]STX to desheathed rat and rabbit sciatic nerves after nerve section. The uptakes in the cut nerves are expressed as fractions of the uptake in control contralateral nerves. (B) Family of Na^+ currents recorded from rabbit cultured Schwann cell evoked by 10 mV depolarizing voltage steps (every second shown) to test potentials of −60 to +100mV. (C) Current-voltage curve obtained from the records shown in Figure A. [Figure A: from Ritchie and Rang (1983), with permission; Figures B and C: from Howe and Ritchie (1990, 1992), with permission.]

the current, which is initially inward, becomes outward at potentials more positive than about +40 mV. This reversal potential corresponds closely with the equilibrium potential for Na^+ calculated from the Na^+ concentrations in the extracellular and pipette solutions. As with axonal sodium channels, the Schwann cell Na^+ channel is blocked in a 1:1 manner by low concentrations of STX, the equilibrium dissociation constant for block being about 2 nM. Parallel STX-binding experiments give a similar value for the equilibrium dissociation constant of binding. Fibroblasts (the other major component of the degenerated nerve trunks of Figure 12-1A) express neither inward currents on depolarization nor do they bind STX. Contrary to the conclusion originally drawn from the experiment of Figure 12-1A, rat cultured Schwann cells do indeed bind STX and express Na^+ currents and STX-binding. However, such expression is very small (on average about 7% of the corresponding values in the rabbit), which probably accounts for it being missed originally.

Sodium Channel Inactivation. It is characteristic of the Na^+ channels that underlie excitability in nerve and muscle that once activated they rapidly inactivate. Indeed, Na^+ channels in such excitable tissue display multiple components of inactivation, that can be distinguished by their different kinetics with inactivation time constants ranging from fractions of a millisecond to many seconds (Howe and Ritchie, 1992, for references). As in the axon, Na^+ channels in Schwann cells can also exist in at least three different inactivated states.

The first two components of inactivation in Schwann cell Na^+ channels are fast; and steady-state inactivation is well described by an h_∞ curve that is quite similar to that of the associated myelinated axon (Chiu et al., 1979). The Hodgkin-Huxley equations predict a single exponential decay of the Na^+ current. However, as Chiu (1977) has shown, this decay in myelinated nerve is in fact biexponential. The onset of inactivation in Schwann cell Na^+ channels is similarly biexponential. Thus the decay of Na^+ current is best fitted by two exponential components (I_{h1} and I_{h2}), one with a time constant (τ_{h1}) of about 0.5 ms and a second with a time constant (τ_{h2}) about five times as large. Although the amplitude of the slower h_2 component is quite small (about 5% of the total) it is consistently found (Howe and Ritchie, 1992). In addition, there is a third inactivated state (I_{hs}) into which the channels enter very slowly on depolarization with a time constant (τ_s) of several seconds.

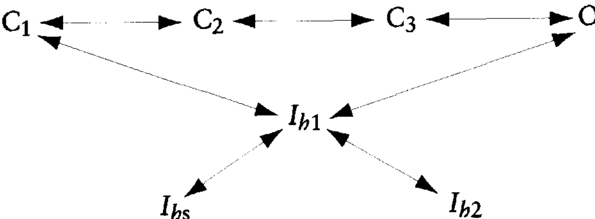
Recovery from this latter inactivated state, though faster than its onset, is still slow, the time constant at −120 mV being 1.2 to 1.5 seconds. Table 12-1 shows the value for the onset and offset time constants as well as the steady-state value of the ampli-

TABLE 12-1. *Schwann Cell Inactivation Time Constants*

	Onset (fast)		Onset (slow)	Offset (slow)	
Potential (mV)	τ_{h1} (msec)	τ_{h2} (msec)	τ_{hs} (msec)		A_s (%)
0	0.47	2.7	6580	1200	0.80
−20	1.11	5.8	–	–	–
−50	(5.0)	—	6980	1160	0.56
−70	—	—	6980	1480	0.36

Mean time constants for the onset of fast (τ_{h1}, τ_{h2}) and slow (τ_{hs}) inactivation at membrane potentials of 0, −20, −50 and −70 mV and for the recovery (at −120 mV) from slow inactivation at holding potentials of 0, −50 and −70 mV. A_s gives the fraction of the channels that are in the slow inactivated state at these potentials. (From Howe and Ritchie, 1992).

tude of this slow component (A_s). Interestingly, at −70 mV (the potential at which Schwann cells are often held in patch-clamp experiments) nearly 40% of the channels are in this slow inactivated state and would not be recruited by the usual brief (100 ms) prepulse to −120 mV before a test pulse. Based on these and other experiments the following model has been proposed for the balance between the closed (C), open (O), and inactivated (I_{h1}, I_{h2}, I_{hs}) states (Howe and Ritchie, 1992).



These results indicate that Na^+ channels in Schwann cells can exist in at least three different inactivated states; and they provide further evidence for the similarity between satellite cell Na^+ channels and the Na^+ channels in their associated axons. Whether yet other inactivated states, with inactivation time constants of minutes also exist in Schwann cells as they do in myelinated nerve and muscle (Fox, 1976; Almers et al., 1983) has not been determined; but such states might account for some of the unavailability of Na^+ channels in electrophysiological experiments.

Although the amplitude of the second fast-inactivating component I_{h2} is normally relatively small, its amplitude relative to that of the I_{h1} component is greatly increased in the decay of sodium currents recorded from cells exposed to internal iodate or to venom from the scorpion *Leiurus* (Howe and Ritchie, 1992). These agents not only alter the relative amplitudes of the two components but also increase τ_{h1} and τ_{h2}. Again, these findings are similar to those found in axonal Na^+ channels (Schmidtmayer, 1985; Neumcke et al., 1987).

Sodium Channel Turnover in Schwann Cells. One speculation for the function of Schwann cell Na^+ channels (see below) is that in the intact animal they are indeed expressed in Schwann cells (where they have no direct function), but are ultimately transferred to (and used by) the axolemma (Gray and Ritchie, 1985; Shrager et al., 1985). The need for such a supplementary mechanism might be particularly acute if the turnover of the channels in the membrane were fast.

An estimate of the lifetime of the sodium channels can be obtained by exposing Schwann cells in culture either to proteolytic enzymes (such as Dispase-collagenase) or to inhibitors of glycosylation (Ritchie, 1988). The former destroys STX-binding capacity, which gradually reappears with an exponential time constant of about 3.1 days; exposure to the latter leads to a progressive exponential fall in STX-binding capacity—again with a time constant of about 3.1 days (Figure 12-2). The assumption that the steady-state density of Schwann cell Na^+ channels is maintained by a constant synthesis of channels in the face of a rate of loss from the membrane proportional to the amount of channel already present leads to the conclusion that these channels have an average lifetime of about 3.1 days, which corresponds with the half-life of 2.1 days. This time is the same order of size as the value of 26 hours for the half-life of Na^+ channels in neuroblastoma cells (Waechter et al., 1983) and value

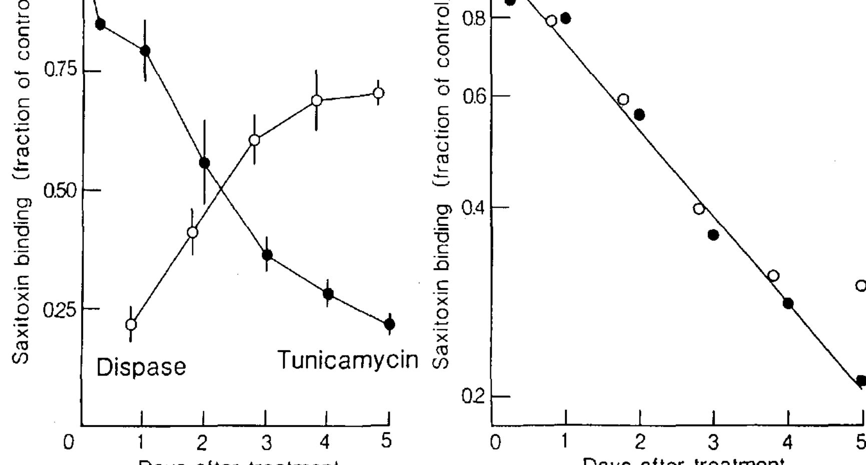


FIG. 12-2. (A) Sodium channel lifetime. The uptake of [^{3}H]STX by rabbit-cultured Schwann cells following exposure either to proteolytic enzymes (dispase-collagenase) (*open circles*) or to tunicamycin (*filled circles*). (B) Same data as in Figure A plotted semilogarhythmically. [From Ritchie (1988), with permission.]

of 2 days found by Schmidt and Catterall (1986) for Na^+ channels in cultured rat neurons. Clearly, a rapid turnover would impose a large metabolic load on the neuron if all the channels in the axon had to be made in the cell body. It has been calculated, for example, that the smallest neuron in the central nervous system might be required to replace as much as 8% of its total protein mass as sodium channels daily (Ritchie, 1988); this replacement would seemingly present a not inconsiderable metabolic load, which would be even greater in remyelinating and regenerating remyelinating nerve where there are many more nodes per unit length of nerve.

Cytoplasmic Sodium Channels. The STX-binding experiments (see Figure 12-1A) indicate that there are about 80,000 binding sites per cell (Howe and Ritchie, 1990). However, even with the maximum sodium current then recorded in Schwann cells (1.2 nA) Shrager et al. (1985) estimated that each cell contained only about 1000 functional sodium channels, which is very much less than the number of binding sites. However, this estimate (based on the ratio of the peak whole-cell and single channel conductances) implicitly assumes that all the channels are open simultaneously at the peak of the Na^+ current, which may not be the case (Aldrich et al., 1983). A more general approach is to estimate the number of channels by dividing the *charge* carried by the whole-cell current, that is, the time-integral of the current, during a depolarizing epoch by the charge carried by a single channel during a similar depolarizing epoch. On that basis the estimate of the number of electrophysiological Na^+ channels becomes nearly 14,000 per cell (Ritchie, 1986), which is still below that estimated from STX-binding. One factor that contributes to the remaining apparent discrepancy is that Schwann cells have long, narrow processes, which must be largely ignored by the recording system in voltage-clamp experiments and so would not contribute to the peak current recorded. For example, in addition to the capacitance associated with the cell soma there is an additional, almost as large, "slow" capacitance, which, because of its high access resistance, is not properly voltage-clamped (Howe and Ritchie, 1992). A second factor is that the Schwann cells are usually held at −70 mV for patch-clamping so that 40% of the channels, being in the long-lasting inactivated state, cannot contribute to the current. Finally, a good deal of the STX binding may well be to *intra*cellular Na^+ channels that have been synthesized by the Schwann cell but have not yet been inserted into the membrane. Lysing of the cells during the binding experiment (by the centrifugation, for example) would expose these channels. The extent to which this factor might contribute can be readily assessed by comparing the [^{3}H]STX binding in Schwann cells both intact and after homogenization. These experiments show that there are indeed approximately equal pools of plasmalemmal and cytoplasmic STX-binding sites (Ritchie et al., 1990). These various factors taken together bring the two estimates, from electrophysiological and STX-binding experiments, reasonably close together.

Current-Voltage Curves. The current-voltage (*I-V*) curve for rabbit Schwann cells (Figure 12-1C), indicates a peak inward current at about −5 mV; and it reverses near a calculated E_{Na}. While this curve is generally similar in shape to that found in rabbit and rat sciatic axons it is clear that the Schwann cell *I-V* curve is shifted along the voltage axis by about 25 mV in the depolarizing direction relative to the curves obtained from the mammalian nodes of Ranvier. In optic nerve astrocytes (see later) the *I-V* curve is also shifted relative to the neuron it invests (the retinal ganglion cell) but in the *opposite* direction; this shift has been interpreted as reflecting the presence of two sodium channels with two quite different molecular structures (Barres et al., 1989b). While such isoforms of the sodium channels are possible (even likely) it seems dangerous to use a shift along the voltage axis as evidence since such a shift could readily be produced by changes in the fixed charges on the inner or outer plasmalemmal surfaces of different cell types or even of the same cell type at different stages of development. Furthermore, large shifts in the *I-V* curve along the voltage axis are readily produced by drugs (such as iodate or scorpion toxins) that acutely modify Na^+ channel activation and inactivation; such shifts occur too quickly for changes in the molecular structure to happen (Cahalan, 1975; Howe and Ritchie, 1992). Even the act of patching produces a substantial shift in the hyperpolarizing direction (Howe and Ritchie, 1992). It is interesting that in the hippocampus the astrocytic and neuronal *I-V* curves follow the pattern of the Schwann cell and its axon rather than the pattern in the optic nerve insofar as the astrocytic *I-V* curve is shifted in the depolarizing direction relative to its neuron (Sontheimer et al., 1991b).

Are Schwann Cell Sodium Channels Normally Expressed? It has been suggested (Chiu, 1987, 1988) that adult Schwann cells that are attached to myelinated fibers do not normally express voltage-gated Na^+ channels, whereas nonmyelinating Schwann cells do. It has further been suggested that this differential expression of sodium channels (i.e., in myelinating as compared with nonmyelinating Schwann cells) may

be absent in newborn animals, thus accounting for the finding that all Schwann cells cultured from neonatal sciatic nerves yield a Na^+ current (Chiu, 1987, 1988). However, no substantial differences have been noted either in the passive electrical properties (input resistance, cell capacitance, resting membrane potential) or in the active electrophysiological properties (peak Na^+ current, membrane potential, *I-V* curve, h_∞) of the whole-cell or single-channel Na^+ currents in Schwann cells from both sciatic and vagus nerves obtained from both neonatal and adult rabbits (Howe and Ritchie, 1990); in the adult these nerves are predominantly myelinated and nonmyelinated, respectively. On this basis the conclusion has been drawn that the expression of Na^+ channels in myelinating Schwann cells does not differ substantially from that of nonmyelinating Schwann cells, nor does it depend on the state of development. The explanation for the reported inability to record Na^+ currents from freshly dissociated myelinating Schwann cells with the patch-clamp method in the whole-cell mode (Chiu, 1987, 1988) seems to be a consequence of the high input capacitance (reported to be on average 670 pF) in conjunction with a series resistance of 10–20 MΩ (see Howe and Ritchie, 1990).

Arguing against Na^+ channels being present normally is the inability to find Na^+ channels in *patches* formed from rabbit sciatic nerve soon after dissociation (Wilson and Chiu, 1990). However, Na^+ channels in patches are known to be elusive. For example, Barres et al. (1989b) found in type 1 astrocytes that although large inward currents were observed in whole-cell recording, such channels were rarely found in patches formed on the soma. Furthermore, the way cells are prepared is important. For example, the usual acute dissociation procedure virtually abolishes the expression of Na^+ currents in Schwann cells (Ritchie, 1988). After a similar procedure in astrocytes only 10 to 20% of them express Na^+ currents, whereas *all* do so if isolated by a less severe procedure of "tissue printing" (Barres et al., 1990a).

Sodium Channels Are Expressed by Schwann Cells In Vivo. The original STX-binding experiments (Figure 12-1A) showed that Na^+ channels were clearly expressed by Schwann cells *in vivo* in the pathophysiological situation of Wallerian degeneration. Two lines of evidence indicate the presence of Na^+ channels in normal nerve *in vivo*. Ritchie and Rogart (1977) found that the total amount of STX bound to intact rabbit sciatic nerve was about 20 fmole mg^{-1} wet nerve. They estimated (from binding experiments to the nonmyelinated vagus nerve) that the sciatic nonmyelinated fibers (and their associated Schwann cells) bound less than 3 fmole mg^{-1} wet nerve. With an internodal axolemmal density of 25 μm^{-2} and an average nodal density of at most 2000 μm^{-2} (as suggested by electrophysiological experiments), only about 10 fmole mg^{-1} could be bound to axolemma (nodal and internodal) meaning that about half the total binding must have been to Schwann cells (Howe and Ritchie, 1990). Furthermore, these must have been myelinating Schwann cells because binding to the nonmyelinated fibers and their Schwann cells was already included in the 3 fmole mg^{-1} "nonmyelinated" estimate given above.

But perhaps the strongest evidence in favor of *in vivo* expression of sodium channels by myelinating Schwann cells comes from immunoblotting experiments with a polyclonal antibody, 7493, that is directed against purified Na^+ channels from rat brain and which specifically recognizes a 260 kD protein corresponding to the alpha subunit of the Na^+ channel (Ritchie et al., 1990). Sections through both the internodal and perinodal regions of the Schwann cell in rat sciatic nerve clearly show the presence of Na^+ channels (Figures 12-3A, B).

Calcium Channels in Schwann Cells

Ca^{2+} currents have not been observed in cultured Schwann cells from rabbit or rat sciatic nerve, the two preparations that yielded invaluable data on the expression of Na^+ channel in Schwann cells (see above). However, a recent study of "organotypic" cultures from mouse dorsal root ganglia shows the expression of whole-cell Ca^{2+} currents by Schwann cells (Amedee et al., 1991). In this study, both L- and T-type Ca^{2+} channels could be identified pharmacologically and kinetically. Interestingly, channels were only expressed when dorsal root ganglion neurons were present in the culture, suggesting that, in culture, neurons may induce the expression of Schwann cell Ca^{2+} channels as previously also shown for cultured rat cerebral astrocytes (Corvalan et al., 1990).

ASTROCYTES

Three major types of glia exist in the central nervous system: oligodendrocytes and two kinds of astrocytes. Only the latter express Ca^{2+} and Na^+ channels, and their expression depends on various factors, including the subtype of astrocyte, which part of the brain it is from, its association with neurons,

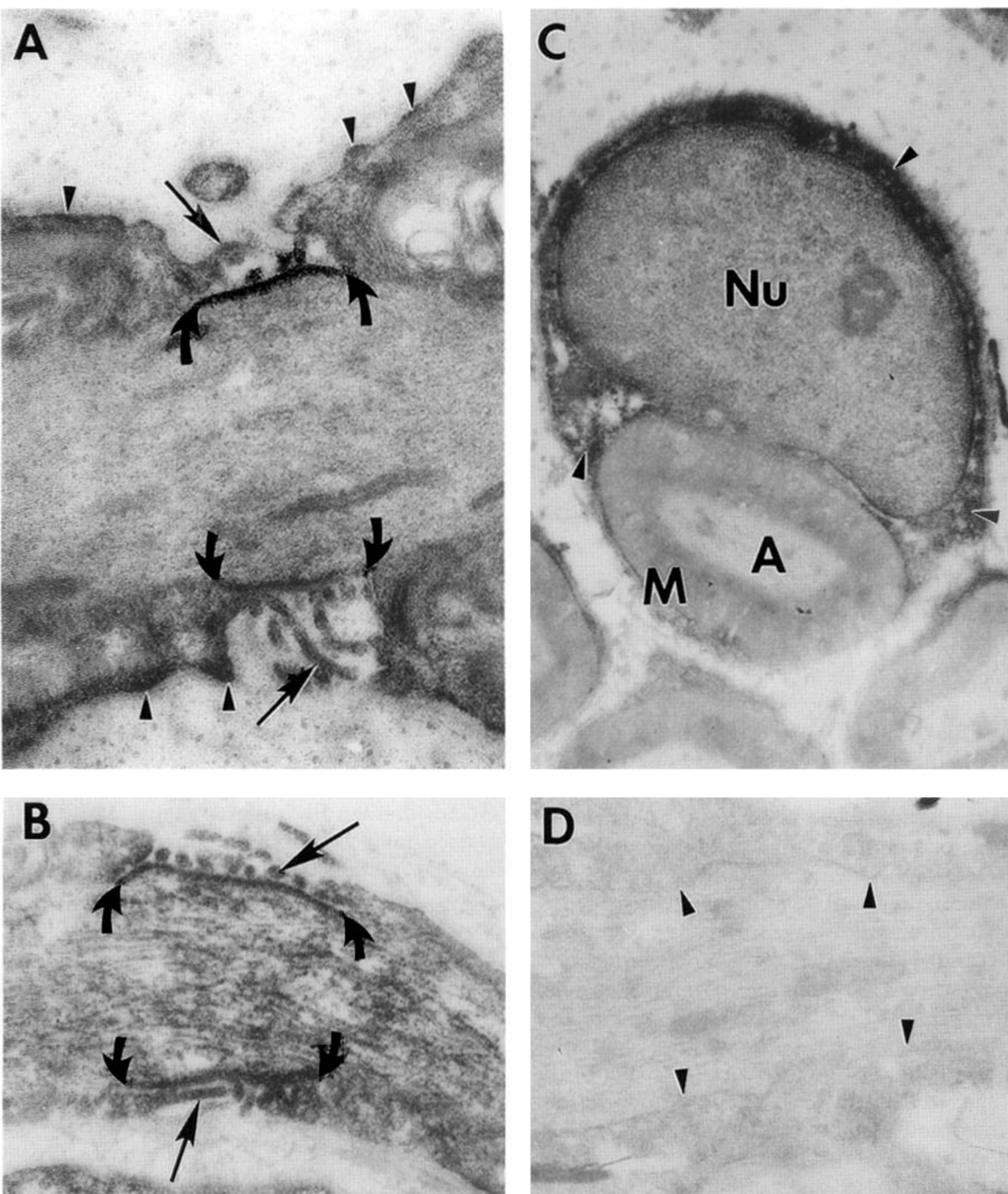


FIG. 12-3. Antibody 7493 immunostaining in Schwann cells of adult sciatic nerve. Nodal axon membrane (*between curved arrows*) of thickly (A) and thinly (B) myelinated nerve fibers display sodium channel immunoreactivity. Perinodal processes (*arrows*) and outer cytoplasmic compartments (*arrowheads*) of Schwann cells exhibit immunostaining. The immunoreactivities are associated both with the cytoplasm and with the Schwann cell plasmalemma. Note the lack of 7493 immunoreactivity in the myelin sheath (*M*) and the paranodal axon membrane. (C) Cytoplasma of myelinating Schwann cell. Note that the Schwann cell nucleus, *Nu*, but not the myelin sheath (*M*) nor the axon (A) display sodium channel immunostaining. (D) Sections incubated with preimmune serum show complete lack of immunoreactivity of nodal axon membrane and Schwann cell processes. (E) 7493 immunoreactivity at node of Ranvier in perinodal astrocyte in rat optic nerve. The axon membrane and subjacent axoplasma at the nodes of Ranvier (*between arrowheads*) exhibits intense immunostaining. Perinodal astrocyte processes (As) are densely immunostained. Note that axon membrane beneath paranodal loops (*PN*) is not immunoreactive. [Figures A–D: from Ritchie et al. (1990), with permission; Figure E from Black et al. (1989), with permission.] *Figure continues*

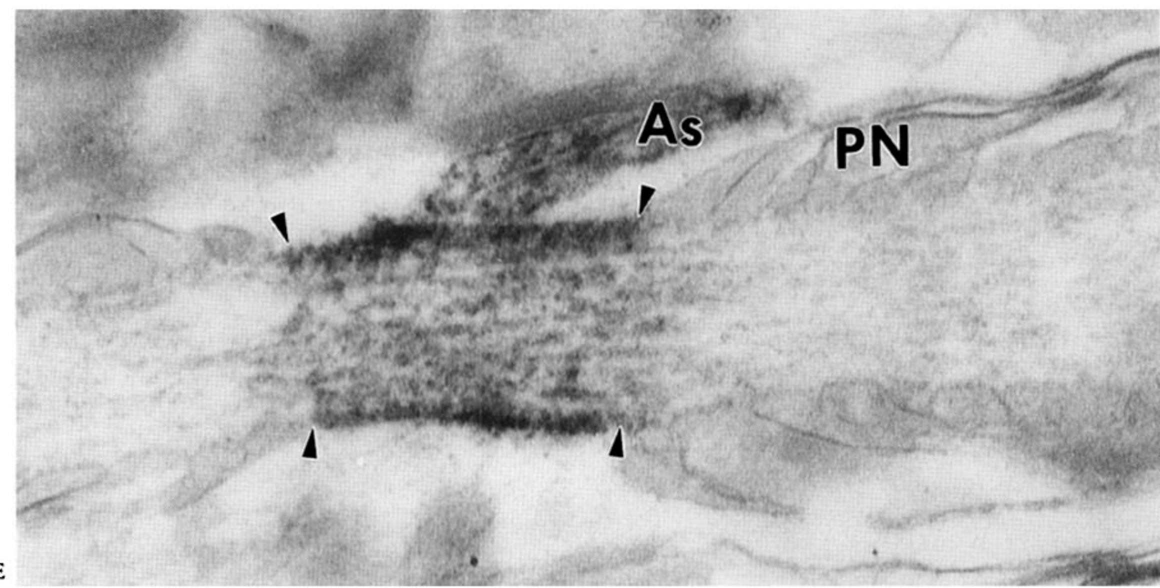


FIG. 12-3. *Continued*

and its developmental stage. These factors will be considered in detail below.

Astrocyte Subtypes

Ramón y Cajal (1913) showed that there are two distinct morphological types of astrocytes in the brain which he termed "protoplasmic" and "fibrous." In intact brain, the fibroblast-like protoplasmic astrocytes are the predominant astrocytes in gray matter, whereas the process-bearing stellate astrocytes resemble white matter astrocytes. These two subtypes of astrocytes are typically represented in cultures from gray and white matter brain areas; and both subtypes often coexist on the same culture. This lineage of astrocytes has been extensively studied in cultures of rat optic nerve, where the two astrocyte subtypes can be further distinguished antigenically (Raff et al., 1983a; Miller and Raff, 1984). Only process-bearing cells bind the monoclonal antibody A2B5; but both subtypes stain positive with astrocyte-specific antibodies to the glial fibrillary acidic protein (GFAP) (Eng, 1985). Interestingly, the two types of rat optic nerve astrocyte develop from different precursor cells; and fibrous astrocytes share a common precursor cell with oligodendrocytes. For simplicity, the two rat optic nerve types were termed type-1 astrocytes ($GFAP^+/A2B5^-$; flat, fibroblast-like) and type-2 astrocytes ($GFAP^+/A2B5^+$; stellate, process-bearing) (Raff et al., 1983a); and this nomenclature has been widely adopted to classify morphologically distinct types of astrocytes regardless of cell origin. Although astrocyte types, distinguished by selective binding of A2B5 antibodies, were subsequently found in rat cerebellar (Levi et al., 1986) and cortical (Levi et al., 1986) cultures, the use of A2B5 antibody for astrocyte classification is not always possible. For example, in rat hippocampal cultures (Sontheimer et al., 1991b), where astrocytes of both morphologies exist, all astrocytes lack A2B5 binding; and in rat spinal cord cultures, where an even greater number of morphologically different types exist, the astrocytes cannot be subdivided on the basis of A2B5 staining (Miller and Szigeti, 1991; Black et al., 1993). Furthermore, A2B5 antibody staining fails to label astrocytes selectively *in vivo* (Miller et al., 1989).

As a result of this heterogeneity of astrocytes, the terms type-1-like and type-2-like astrocytes are frequently used in the literature, referring mostly to morphologically identified astrocytes that comply with the morphological criteria defining protoplasmic (i.e., flat, fibroblast-like) and fibrous (i.e., stellate, process-bearing). Since the terms type-1 and type-2 were introduced for rat optic nerve astrocytes, which are characterized not only by differences in A2B5 binding but also by specific lineage relationships (Raff et al., 1983a, 1983b; Temple and Raff 1985), this terminology is not always applicable and should be used only for rat optic nerve astrocytes. For simplicity, in the following discussion all astrocyte types will be referred to as protoplasmic or as fibrous, emphasizing only their morphological difference without implying different regional origins (e.g., white or gray matter) or unique lineage.

Calcium Channels in Astrocytes

Although voltage-gated Ca^{2+} channels were among the first to be described (MacVicar, 1984) they have been less extensively studied. They are, however, clearly present in astrocytes. Under conditions where Na^+ and K^+ channels are blocked, and where Ca^{2+} ions are replaced by Ba^{2+} ions (which pass more easily through Ca^{2+} channels) action potentials can be evoked by electrical stimulation of cultured rat astrocytes; and they can even occur spontaneously (Figure 12-4A). The Ca^{2+} current depends markedly on prior exposure of the astrocytes to agents that raise the intracellular concentration of cAMP, such as dibutyryl cAMP (dcAMP), norepinephrine, and isoproteranol (MacVicar and Tse, 1988), as well as 8-bromo-cAMP, forskolin, and vasoactive intestinal peptide (Barres et al. 1989a). This current is mostly an L-type Ca^{2+} current (as defined in McCleskey et al., 1986), and is blocked by nifedipine, Co^{2+}, and Cd^{2+}. Relative to the other glial cell currents, the Ca^{2+} current is relatively small (25 to 200 pA) being 1 to 2 orders of magnitude smaller than Na^+ or K^+ currents.

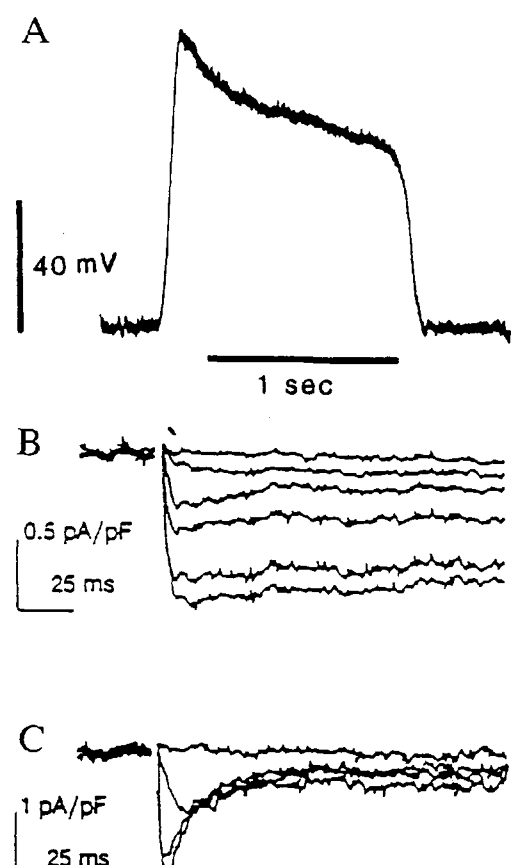


FIG. 12-4. (A) Spontaneous Ca^{2+} action potential in rat astrocyte (in Ba^{2+} Locke solution). [From MacVicar (1984), with permission.] (B, C) L-type and T-type Ca^{2+} currents in type-1 rat optic nerve astrocytes in response to families of depolarizing steps between −60 and +25 mV, and −60 and −5 mV, respectively. [From Barres et al. (1990), with permission.]

Astrocytes in culture, however, usually do not express Ca^{2+} currents. Indeed, MacVicar's original demonstration of astrocytic Ca^{2+} currents probably owed its success to the fact that the cortical astrocytes being studied had been "rounded" by a 2-hour exposure to dcAMP some hours beforehand to facilitate recording. However, factors other than the drug-induced action described above may also be involved. For example, forskolin does not always induce astrocytic Ca^{2+} currents—only in the presence of a few batches (less than 20% of number tested) of the fetal calf serum used in culturing (Barres et al., 1989a). Furthermore, while the Ca^{2+} current is L-type when induced by some lots of serum, with other lots the current is of the more transient T-type. The former activates at −30 mV and inactivates quite slowly, whereas the T-type current activates at about −60 mV and has a rapid voltage-dependent inactivation.

One of the most intriguing findings is that the expression of Ca^{2+} currents by glial cells critically depends on the presence of neurons. Thus, Corvalan et al. (1990) have shown that, whereas no inward currents can normally be detected in pure cultures of rat cortical astrocytes, both L-type and T-type Ca^{2+} currents are expressed when the astrocytes are cocultured with neurons. This dependence on neuronal presence is reminiscent of the similar dependence of Na^+ currents described later for rat optic nerve astrocytes (Figure 12-6).

The method of cell isolation also seems to be important. Thus, although Ca^{2+} currents are normally absent in cultured type-1 rat optic nerve astrocytes, both types of Ca^{2+} current are detected when the astrocytes are acutely dissociated from freshly excised nerve by the new "tissue print" procedure involving treatment with the proteolytic enzyme papain (Barres et al., 1990a). Some cells express just L-type currents (Figure 12-4B), others just T-type currents (Figure 12-4C), while others express both. Even with the tissue print procedure, however, astrocytes prepared from transected (i.e., axon-free) optic nerve do not express Ca^{2+} currents—which might be explained by the neuron-glial interaction just described.

Sodium Channels in Astrocytes

Patch-clamp studies of astrocytes cultured from numerous central nervous system regions [cerebrum

(Bevan et al., 1985; Bevan et al., 1987; Nowak et al., 1987); spinal cord (Sontheimer et al., 1992a; Sontheimer and Waxman, 1992b); optic nerve (Barres et al., 1988, 1989b, 1990a; Sontheimer et al., 1991a); and hippocampus (Sontheimer et al., 1991b)] demonstrate that at least 30% of cultured astrocytes in any given cell preparation express voltage-gated Na^+ currents. These astrocyte Na^+ currents appear to be generally similar to Na^+ currents in neurons with respect to their strong voltage-dependence, rapid channel kinetics (Figure 12-5A), and the blocking action of TTX. Single-channel recordings from cultured rat optic nerve astrocytes show a single-channel conductance of 18 pS (Barres et al., 1990a), which is similar to the conductance of Na^+ channels of excitable cells (Hille, 1992).

A more detailed analysis of astrocyte Na^+ current demonstrates that the two subtypes of astrocyte (protoplasmic and fibrous) express Na^+ currents, which differ from each other in various aspects of their steady-state voltage-dependence, their kinetics, and their channel pharmacology (Figure 12-5). Thus, in astrocytes from rat optic nerve (Barres et al., 1989b; Sontheimer et al., 1991a), hippocampus (Sontheimer et al., 1991b) and spinal cord (Sontheimer et al., 1992; Sontheimer and Waxman, 1992b), two kinds of Na^+ current can be distinguished in terms of their different steady-state inactivation (h_∞) and *I-V* curves (Barres et al., 1989b; Sontheimer et al., 1991a, 1991b, 1992; Minturn et al., 1992; Sontheimer and Waxman, 1992b)). In spinal cord astrocytes, for example (Figure 12-5B), the *I-V* curves for

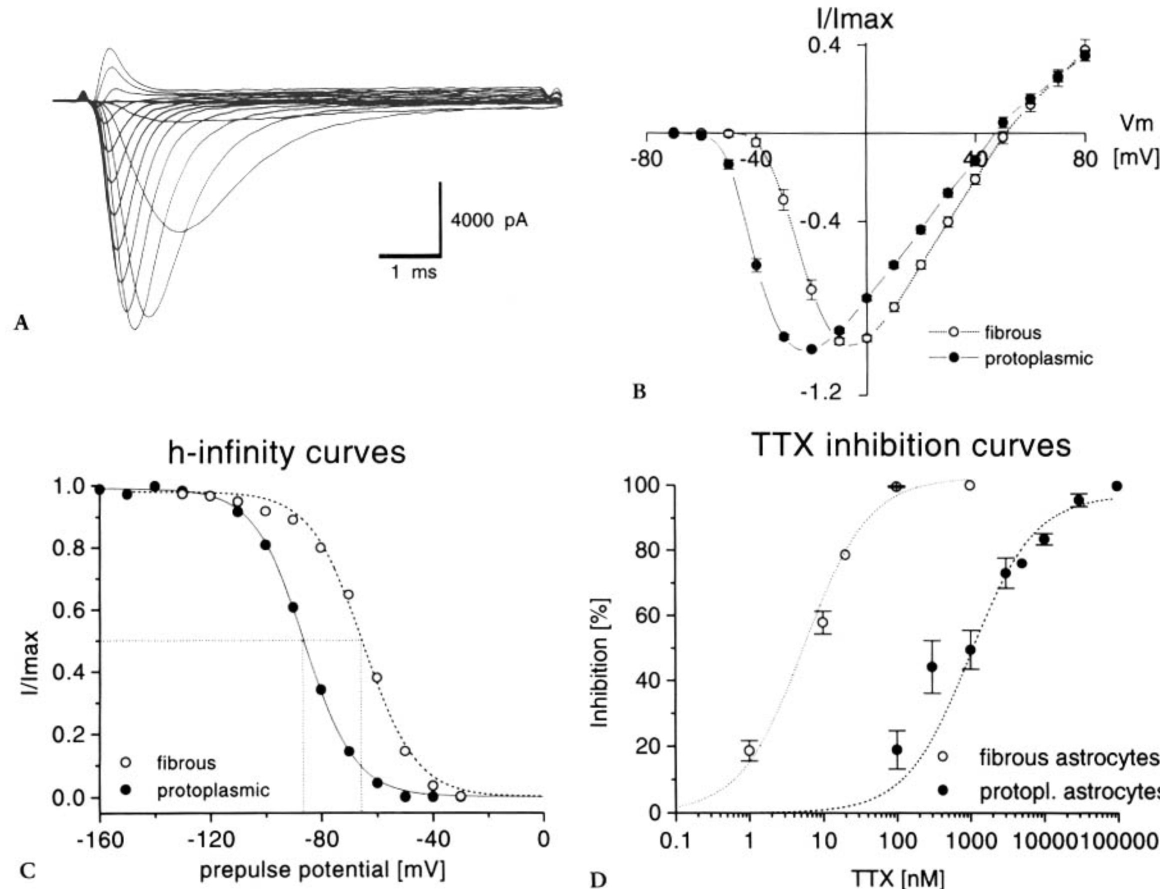


FIG. 12-5. Astrocytes subtypes express Na^+ currents with differing TTX sensitivity, activation and inactivation curves. (A) Na^+ currents expressed by astrocytes are fast, transient currents. Time to peak at 20°C is <800 μs, and current kinetics show overall resemblance of Na^+ currents in excitable cells. (B) Na^+ currents recorded in stellate and protoplasmic, spinal cord astrocytes differ in their *I-V* curves, in that the peak and activation threshold of the *I-V* curve is 16 mV more positive in fibrous cells than in protoplasmic cells. (C) Steady-state current inactivation differs similarly and shows a 20-mV positive shift fibrous astrocytes. Data in Figures C and D were normalized means from at least 20 recordings each, and the data were fit to Boltzmann equations. (D) The two astrocyte subtypes differ in their sensitivity to TTX. Na^+ currents in fibrous astrocytes are highly TTX-sensitive (K_d = 5.7 nM) whereas protoplasmic astrocytes are comparatively TTX-resistant (K_d = 1000 nM). Data displayed was obtained from 39 cells and means were fit to Langmuir binding isotherms (*dotted and dashed line*). [From Sontheimer et al. (1992a) and Sontheimer and Waxman (1992), with permission.]

fibrous astrocytes are shifted along the voltage axis in the depolarizing direction relative to those of protoplasmic astrocytes; and their activation curves are similarly shifted (on average by about 16 mV). The two astrocyte subtypes also differ in their steady-state inactivation (h_∞) curves; thus the midpoint of the curve for fibrous cells is at about -65 mV, whereas for protoplasmic astrocytes it is close to -90 mV (Figure 12-5C).

The response to TTX further emphasizes this apparent dissimilarity of Na^+ currents in the two astrocyte subtypes. Thus, as Bevan et al. (1985) first noted, Na^+ currents in rat cerebral astrocytes are relatively insensitive to TTX, micromolar concentrations being required for channel block; and similar comparatively low TTX sensitivity for Na^+ channels exists in cultured mouse cerebral astrocytes (Nowak et al., 1987).

However, this relative insensitivity to TTX applies only to protoplasmic astrocytes. Fibrous astrocytes express channels that are blocked by *nano*molar concentrations of TTX, as in neuronal and Schwann cells channels. Thus, in the spinal cord where fibrous and protoplasmic astrocytes are both expressed, both kinds of Na^+ channel, TTX-sensitive (TTX-S) and TTX-resistant (TTX-R) Na^+ channels, respectively are found. Each channel type is associated with its own morphological astrocyte type, TTX-R with protoplasmic, and TTX-S with fibrous astrocytes (Figure 12-5D). For complete block, TTX-R Na^+ channels require micromolar TTX concentrations (K_D = 1000 nM), whereas TTX-S channels require nanomolar concentration (K_D = 6 nM). Thus, in addition to differences in steady-state activation and inactivation parameters, the two astrocyte types also differ in their Na^+ channel pharmacology. Whether these differences also imply differences in the Na^+ channel protein expressed remains to be shown.

It should be noted that, although the electrical activity of rat cerebral astrocytes is relatively insensitive to TTX, these cells also express a high-affinity site (K_D ~ 2 nM) for saxitoxin, which is a specific marker for Na^+ channels (Bevan et al., 1985). One explanation would be that both TTX-R and TTX-S Na^+ channels are present in the astrocyte population, the latter being expressed, however, to a relatively small extent.

Based on differences on the voltage dependence of activation and inactivation, it has been postulated (Barres et al., 1989b) that protoplasmic rat optic nerve astrocytes (type-1) express a unique "glial" molecular form of Na^+ channel, whereas fibrous rat optic nerve astrocytes (type-2) express a "neuronal" type. However, as discussed earlier in conjunction with a similar phenomenon in Schwann cells, these steady-state parameters are influenced by numerous factors, including surface charge and toxins, so caution should be exercised in the use of such parameters for distinction. A more reliable indication that multiple isoforms of the Na^+ channel are present comes from the widely different sensitivities of fibrous and protoplasmic astrocytes to TTX. Sensitivity to TTX appears to be determined by the primary amino acid sequence of the channel protein itself (Noda et al., 1989; Yang et al., 1992). Thus, the observed difference in TTX pharmacology between the two astrocyte subtypes is more compelling in suggesting that protoplasmic astrocytes do indeed express distinct Na^+ channel proteins.

A partial clone of a putative glial Na^+ channel gene (Na-G) has recently been characterized, and the amino acid sequence deduced from this cDNA indicates that this glial Na^+ channel represents a separate molecular class within the mammalian Na^+ channel multigene family (Gautron et al., 1992), supporting the notion that a distinct astrocytic Na^+ channel exists. Nevertheless, molecular cloning, and subsequent *in vitro* expression and functional characterization of an astrocytic Na^+ channel gene is required to provide unequivocal proof.

Regional and Developmental Heterogeneity in Astrocyte Na^+ Channel Expression. In reviewing the literature of Na^+ channel expression in astrocytes, (predominantly originating from four laboratories) one is astonished at the diversity of conditions used to obtain *in vitro* preparations for study. These differences include the use of two rodent species (mouse and rat), four different brain regions (cortex, hippocampus, optic nerve, spinal cord), and numerous differences in the culture substrates and media used to maintain the astrocytes *in vitro*. Additionally, and seemingly most importantly, the time *in vitro* after which recordings were obtained and the age of culture derivation varied. Those differences are likely responsible for the apparent heterogeneity reported.

Regional Heterogeneity and Subtype Heterogeneity. In most culture preparations only subpopulations of astrocytes (typically, approximately 30% of cells) express voltage-activated Na^+ channels (Nowak et al., 1987; Sontheimer et al., 1991a, 1991b). This is also true for astrocytes that are antigenically and morphologically identical. Thus only 30 to 40% of protoplasmic (type 1) rat optic nerve astrocytes show measurable Na^+ currents at 14 to 20 DIV (Sontheimer et al., 1991a). Similarly, less than 30% of hippocampal astrocytes show Na^+ current expression after 10 to 20 DIV (Sontheimer et al., 1991b). Although in rat optic nerve and spinal cord the two morphological subtypes of astrocytes each express

its specific Na^+ current type as discussed above (Barres et al., 1989b; Sontheimer et al., 1991a, 1992b; Minturn et al., 1992; Sontheimer and Waxman, 1992b), both protoplasmic and fibrous astrocytes express identical Na^+ current types in hippocampal cultures; but the type of Na^+ current changes during *in vitro* (Sontheimer et al., 1991a) and *in vivo* (Sontheimer et al., 1992b) development.

Developmental Heterogeneity. In rat optic nerve, hippocampus and spinal cord astrocytes, Na^+ channel expression changes during *in vitro* development, and depends critically on the age of culture derivation. In contrast, cultures obtained from neonatal rat optic nerve, contain almost exclusively protoplasmic astrocytes (Raff et al., 1983a; Sontheimer et al., 1992a). Rat optic nerve astrocyte cultures obtained from P7 animals contain both morphological subtypes of astrocytes because fibrous (type-2) astrocytes develop in addition in the second postnatal week (Raff et al., 1983a, 1983b). Recordings obtained from these preparations are quite different from each other: Protoplasmic (type-1) rat optic nerve astrocytes from PO animals do not express Na^+ channels until 5 DIV (Sontheimer et al., 1991a); and, interestingly, astrocytes derived from P7 rat optic nerve show their highest level of Na^+ channel expression immediately after plating and decline thereafter. Na^+ currents are virtually absent in cells cultured for more than 14 DIV; and they are not detectable at all in cultures of adult rat optic nerve (H. Sontheimer, unpublished observations). In contrast, fibrous rat optic nerve astrocytes in the same cultures always show constant levels of Na^+ channel expression regardless of time in culture. As in rat optic nerve astrocytes, protoplasmic rat spinal cord astrocytes obtained from embryonic (Sontheimer, unpublished observations) or neonatal cords lack Na^+ channel expression until 5 DIV, show a peak in channel expression at 7 to 8 DIV, and there is a decline in channel density thereafter (Sontheimer et al., 1992a). Fibrous spinal cord astrocytes, like their counterpart in rat optic nerve, are more invariant; and they always show Na^+ channel expression at similar densities. These observations from spinal cord and rat optic nerve suggest that Na^+ channel expression in protoplasmic astrocytes is a transient phenomenon, probably occurring during the second and third week of postnatal development. In contrast Na^+ channels are always expressed by stellate astrocytes, independently of development. The timecourse of Na^+ channel expression in protoplasmic astrocytes may correlate with cell proliferation and the formation of functional gap-junction coupling, a feature that occurs in protoplasmic, but not fibrous, astrocytes (Sontheimer et al., 1990). In hippocampal astrocytes, Na^+ channel expression correlates with the absence of gap-junction coupling: coupled cells lack expression (Sontheimer et al., 1991c). In summary, therefore, it is important to examine channel expression in the context of cell development.

Expression of Astrocyte Sodium Channels is Under Neuronal Control. In the intact brain numerous cell types are in close proximity, sharing a common environment through their common boundaries with the extracellular space. In particular, astrocytes and neurons and their processes can be in close apposition. For example, in rat optic nerve, astrocyte endfeet reach into nodes of Ranvier and are directly apposed to the axolemma (Black et al., 1989). However, patch-clamp recording is almost always done on cultured astrocytes that are deprived of this potentially important contact with surrounding neurons. A number of observations indicate that astrocyte ion channel expression, and particularly the expression of Na^+ channels, is in fact influenced by their neuronal surroundings or by the presence or absence of axons.

Astrocytes cultured from animals in which the optic nerve axons have degenerated following neonatal monocular enucleation show little or no expression of Na^+ currents, whereas Na^+ currents are pronounced in control astrocytes obtained from intact nerves (Minturn et al., 1992) (Figure 12-6). The deprivation of axonal contact not only drastically reduces the density at which Na^+ channels are expressed but also reduces the percentage of cells that show any channel expression at all. As with enucleation, total nerve transsection almost completely abolishes Na^+ currents in astrocytes dissociated from these optic nerves; and this effect can be partially restored if cells are cocultured with neurons (Barres et al., 1990a).

Both procedures described above, enucleation, and nerve transection, lead to a similar complete loss of fibrous (type-2) astrocytes in rat optic nerve. Thus, such studies are limited to Na^+ channel expression in protoplasmic (type-1) astrocytes.

In contrast to the observations on rat optic nerve astrocytes, which suggest that the presence, and/or activity, of axons have a positive modulatory influence on astrocyte Na^+ channel expression, downregulation of Na^+ channel expression by neurons is observed for both subtypes of spinal cord astrocytes (Thio et al., 1993). Thus, coculture of spinal cord astrocytes with dorsal root ganglion neurons, leads within 2 days to a more than 70% reduction in Na^+ current density compared to that of matched control cultures grown in the absence of neurons. Culturing

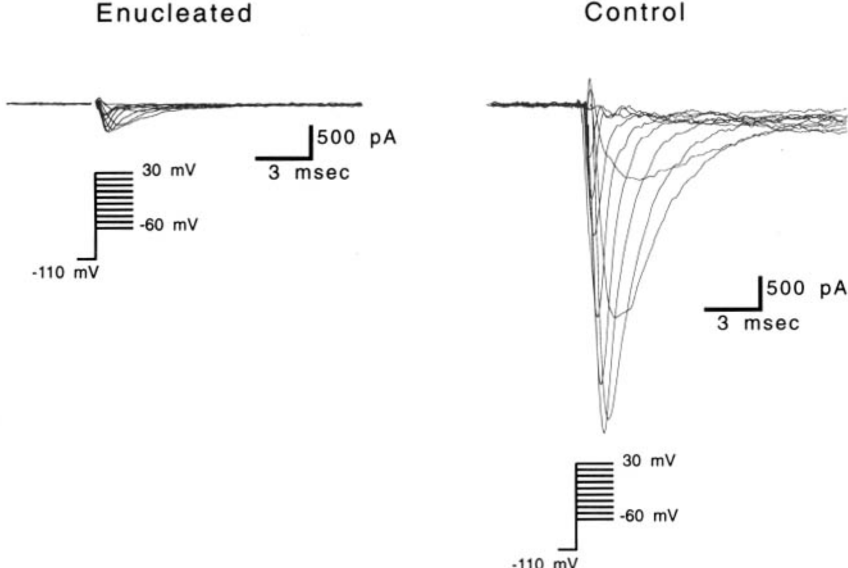


FIG. 12-6. Neuronal influence on astrocyte Na^+ channel expression. Astrocytes cultured from optic nerve following monocular enucleation showed markedly reduced levels of Na^+ currents as compared to cells cultured from control nerves. Enucleation reduced the peak Na^+ currents and the percentage of cells expressing any measurable Na^+ currents. The examples displayed are the largest Na^+ currents recorded in the two groups, and were displayed to scale for better comparison. [From Minturn et al. (1992), with permission.]

cells in the presence of neuron-conditioned medium has a similar, although less potent effect.

It is not known whether the differences observed in the modulatory role of neurons on astrocyte Na^+ channel expression between spinal cord and optic nerve represents an intrinsic, regional difference of astrocytes, or whether the different neuronal/axonal populations account for them. Nor are the means by which neurons control the expression of astrocyte ion channels known; but the potential of neuron-conditioned media to mimic effects of neurons or their processes suggests that a soluble factor must serve as signal.

The above-described influences on the expression of ion channels require hours or days to occur, and are likely to regulate the synthesis of channel protein. Neuronal influences on a much faster time-scale have been observed in frog optic nerve (Marrero et al., 1989). Here, Na^+ currents in glial processes contributing to the glial limitans are transiently facilitated in response to nerve impulses seconds after stimulation, suggesting that fast acting modulatory mechanisms must exist in addition to the slower regulation of channel expression. Again, the means by which this modulation is mediated is unknown.

Astrocytes In Vivo Express Sodium Channels. Almost all studies characterizing voltage-dependent Na^+ channel expression in astrocytes have utilized patch-clamp recordings from astrocytes *in vitro* (cell culture or acutely dissociated cells). In contrast, the classical electrophysiological studies of glial cells by Kuffler and colleagues (Kuffler and Potter, 1964; Kuffler et al., 1966; Orkand et al., 1966), which utilized *in situ* preparations, did not indicate the presence of any voltage-dependent conductances. The apparent discrepancy in these observations raises the question as to whether channel expression may be an epiphenomenon peculiar to cell culture, or perhaps a result of differing recording techniques. A number of laboratories have addressed this question in different ways.

1. Using a polyclonal antibody (7493) directed against rat brain Na^+ channels, Black et al. (1989) have demonstrated the presence of Na^+ channel immunoreactivity in astrocyte processes at nodes of Ranvier in the intact rat optic nerve (Figure 12-6).
2. Barres and colleagues (1990a) have utilized a new "tissue print" dissociation procedure, which allowed them to isolate astrocytes from optic nerve with most of their processes intact and obtain patch-clamp recordings. These cells, like astrocytes in culture, express voltage-activated Na^+, K^+, and Ca^{2+} currents, suggesting that the channels mediating these currents are also expressed *in vivo*. However, as in other dissociation procedures, enzymes were still used to isolate cells, and the recordings were necessarily made in the absence of the natural neuronal environment of the astrocytes. Thus it is still possible that the cultured cells show responses not observed in the intact brain (where, for example, the expression, and/or activity, of ion channels could be suppressed by adjacent neurons).
3. A number of laboratories have started to apply patch-clamp studies to more intact tissue preparations such as whole-mount retina and isolated brain slices. The first such studies on glial cell have demonstrated that voltage-activated Na^+ channels are expressed in glial precursor cells in corpus callosum

slices (Berger et al., 1991) and in identified astrocytes in hippocampal slices (Sontheimer and Waxman, 1992a, 1993; Steinhäuser et al., 1992) (Figure 12-7A). Similarly, astrocytes in the intact rabbit retina have been shown to express Na^+ channels with biophysical characteristics of the Na^+ currents expressed in protoplasmic optic nerve astrocytes (Clark and Mobbs, 1992).

These studies, particularly those *in situ,* seem to dismiss concerns that Na^+ channel expression in glial cells is an artifact of cell culture.

Astrocyte Sodium Channels Are Not Involved in the Generation of Action Potentials. Na^+ channels in excitable cells are responsible for the upstroke of the action potential (Figure 12-7B, D); and no other functions of voltage-activated Na^+ channels are known. Consequently, the expression of such channels by astrocytes raises the question of their ability to generate action potentials. However, astrocytes express Na^+ channels at a density that is typically at least 1 to 2 orders of magnitude lower than in excitable cells, being well below 1 channel per square micrometer (Table 12-2) as compared to tens or hundreds of channels per square micrometer for neurons. Furthermore, the peak Na^+ conductance in neurons is at least equal to the peak K^+ conductance, whereas in astrocytes it is usually 5- to 10-fold smaller than the K^+ conductance (Figure 12-7A, B). This leads to a situation in which the astrocyte resting potential is stabilized in a "voltage-clamp-like" way by its high K^+ conductance, so preventing the activation of Na^+ currents.

In isolated spinal cord astrocyte cultures, proto-

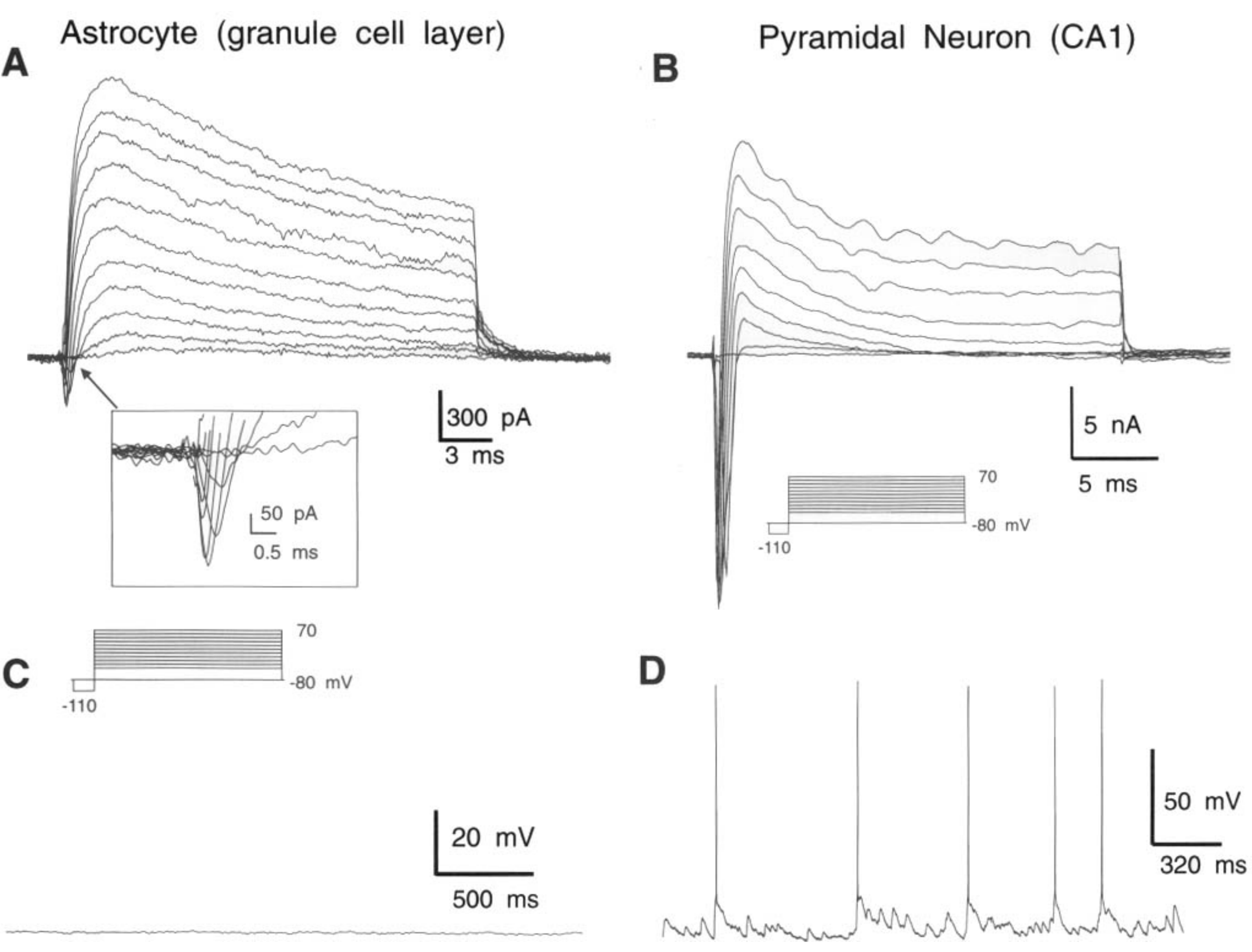


FIG. 12-7. Comparison of typical whole-cell current responses in a representative hippocampal neuron and astrocyte recorded in brain slice. Currents were elicited in response to depolarizing voltage steps to the potentials indicated. Recordings were obtained with a KCl-containing pipette solutions, to allow both Na^+ current and K^+ current activation. In the neuron (*B*) the relative proportion of Na^+ to K^+ currents shows a dominance of Na^+ currents, whereas in the astrocyte (*A*) outward K^+ currents were much larger than inward Na^+ currents. While the neurons displayed spontaneous synaptic activity and fires action potentials (*D*) the astrocyte did not (*C*). [From Sontheimer and Waxman (1993), with permission.]

TABLE 12-2. *Sodium Channel Densities*

Neurons		Astrocytes	
Squid giant axon (Conti et al., 1975)	330	RON Type 1 (Barres et al., 1989)	<0.35
Frog node (Conti et al., 1976)	1900	(Sontheimer et al., 1991a)	<0.25
Rat node (Neumcke and Staempfli, 1982)	700	RON Type 2 (Barres et al., 1988)	<0.1
		(Sontheimer et al., 1991a)	0.25
Rabbit myelinated node (Ritchie and Rogart, 1977)	10,000	Rat hippocampus (Sontheimer et al., 1991b)	<0.9
Mouse spinal cord culture 2 DIV (MacDermott and Westbrook, 1986)	<2	Cerebrum (Sontheimer, unpublished)	<0.4
14-21 DIV (Catterall, 1984)	50–75	Spinal cord (Sontheimer et al., 1992)	8
Chick DRG culture (Carbone and Lux, 1986)	2–20		
Rat hippocampus culture (Sontheimer et al., 1991)	5		

Values represent channels/ μm^2.

plasmic astrocytes can transiently express very high densities of Na^+ channels during the second week of *in vitro* development (Sontheimer et al., 1992a), with channel densities as large as typically seen in cultured neurons. Indeed action potential-like responses can be recorded from such cells under current-clamp. Potentials more negative than −70 mV are required to elicit such responses to current injection; and the cells never fire spontaneous action potentials. In fact, a mismatch of resting potential (−30 to −40 mV) and steady-state Na^+ current inactivation (h_∞) curve midpoint, (−90 mV) must inactivate the majority of Na^+ channels and render them nonfunctional. As demonstrated above, Na^+ channel expression in these cells is suppressed by the presence of neurons. *In vivo* channels densities may be far too small to generate action potentials, and no spontaneous electrical activity can be recorded in astrocytes *in situ* (Figure 12-7C).

In summary, therefore, it is highly unlikely that astrocytes use Na^+ channels to generate action potentials (see also Chapter 20).

Oligodendrocytes

Although compelling evidence exists that oligodendrocytes share a common precursor cell with stellate astrocytes (Raff, 1989), oligodendrocyte channel expression differs from that of their astrocyte siblings. Thus, as the bipotential glial progenitor cell that expresses voltage-activated channels Na^+ (Sontheimer et al., 1989b; Barres et al., 1990b), Ca^{2+} (Verkhratsky et al. 1990), and numerous types of K^+ channels (Sontheimer et al. 1989b; Barres et al., 1990b) makes a commitment to differentiate into an oligodendrocyte, it abandons the expression of voltage-activated Na^+ and outwardly rectifying K^+ channels (Sontheimer and Kettenmann, 1988; Sontheimer et al., 1989b). As in developing neurons (Konnerth et al., 1987), however, both glial precursor cells and oligodendrocytes (Sontheimer et al., 1989a) express a different class of Na^+ channel, which is insensitive to TTX and largely insensitive to voltage. These pH-activated Na^+ channels [Na_H^+] are gated by a step-acidification of their extracellular milieu (Krishtal and Pidoplichko, 1980). A pH step from pH 7.9 to pH 6.7 induces a large, transient, comparatively slow inward current in glial precursor cells, oligodendrocytes, and neurons, but not in protoplasmic astrocytes or Schwann cells (Figure 12-8). The pH-activated Na^+ currents are potentiated by reduced $[Ca^{2+}]_o$ and show a "run-down" with repeated pH challenges (Sontheimer et al., 1989a). Na_H^+ can be coexpressed with TTX-S voltage-dependent Na^+ channels in precursor cells and neurons, and its expression precedes the expression of voltage-activated channels (Grantyn et al., 1989) during neuronal development. A functional role has not yet been identified for Na_H^+ in oligodendrocytes and neurons.

Glial Precursor Cells

Much attention has been paid to studying electrophysiological properties of cells developing along the oligodendrocyte lineage from the bipotential oligodendrocyte-type 2 *a*strocyte (O-2A) progenitor cells to differentiated oligodendrocytes (Sontheimer et al., 1989b; Barres et al., 1990b). Some of these studies have utilized stage-specific monoclonal anti-

bodies that allow the differentiation of at least two developmental stages of precursor cells and two stages of oligodendrocytes (Sontheimer et al., 1989b); and these studies have demonstrated dramatic changes in channel complement during cell development. In mouse cerebrum (Sontheimer et al., 1989b) and rat optic nerve (Barres et al., 1990b), precursor cells are characterized by a large complement of channels, including TTX-S Na^+ channels and 3 types of K^+ channels (K_d, K_a, and K_{ca}). Mouse cerebrum precursor cells may also express L- and T-type Ca^{2+} channels (Verkhratsky et al., 1990b). As precursor cells differentiate to oligodendrocytes, these channels are lost and replaced by inwardly rectifying K^+ channels (Sontheimer et al. 1989b) which constitute the major channel type in differentiated oligodendrocytes *in vitro* (Barres et al., 1990b; Sontheimer et al., 1989b) and *in situ* (Berger et al., 1991; Sontheimer and Waxman, 1993).

WHAT IS THE FUNCTION OF SATELLITE CALCIUM AND SODIUM CHANNELS?

Calcium Channels

The central role of transmembrane Ca^{2+} currents in so many physiological processes is now well established for a wide variety of tissues; one must presume, therefore, that such currents play similar important roles in satellite cells. What these specific roles are, however, remains unclear. This is partly because even though the channels are present in the membrane of astrocytes and Schwann cells *in vivo* the existence of Ca^{2+} *currents* is open to question. For there is the problem for both T-type and L-type Ca^{2+} channels (shared with Na^+, K^+, and Cl^- voltage-gated channels) that their apparent activation thresholds (−60mV and −30mV, respectively) are considerably more depolarized than the reported astrocytic resting potential (of about −80mV). When would they ever be open to any significant extent? The answer may be that not all astrocytes have resting potentials of the order of −80 mV (at least not all of the time). Certainly during *intense* electrical neuronal activity, the depolarization produced might well be sufficient to activate these channels. Under certain spreading-depression-like pathological conditions such as associated with ischemia, trauma, anoxia, or hypoglycemia, extracellular K^+ concentration can rise to 80 mM (Somjen, 1979 for review), and under those conditions depolarizations would certainly be large enough to activate glial Ca^{2+} channels.

In spite of these gaps in our knowledge, there can be little doubt that changes (even small ones) in cytosolic Ca^{2+} concentrations must be important not just for the household functions of the glial cell but also in its interaction with neurons. Indeed, the coculturing experiments (Corvalan et al., 1990) suggest that experiments with purified cultures of astrocytes may not be best for determining the functions *in vivo*, where the glial cells are in intimate contact with neurons. But speculation on these points requires a more solid base provided by future experimentation.

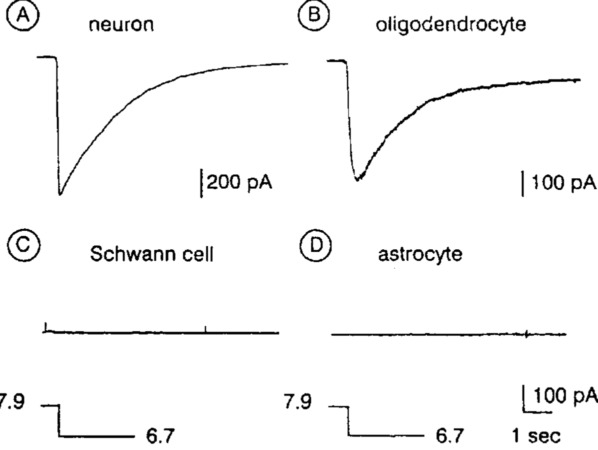
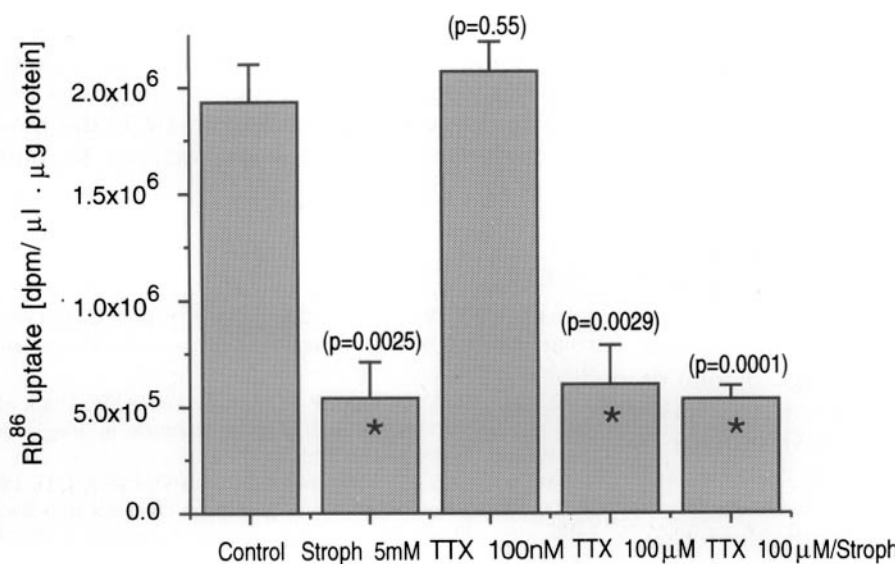


FIG. 12-8. Proton activated Na^+ channels are expressed in oligodendrocytes. Neurons (A) and oligodendrocytes (B) in contrast to Schwann cells (C) and astrocytes (D) express proton-activated Na^+ channels. Stepwise changes of the external pH from 7.9 to 6.7 were applied and resulted in the inward currents displayed. All recordings were taken in the presence of 1 μM TTX at a holding potential of −70 mV. Extracellular Ca^{2+} was 0.1 mmol, Mg^{2+} was omitted.

Sodium Channels

In astrocytes the membrane resting potential is quite high, being −75 to −85 mV (Ballanyi et al., 1987). In Schwann cells and glial precursor cells, however, the resting potential (in mouse, rat, and rabbit) is much smaller, between −40 mV and −50 mV (Konishi, 1989; Howe and Ritchie, 1990; Verkhratsky et al., 1991). This means that the Schwann cell Na^+ channels cannot be involved in any regenerative action such as nerve impulse conduction because they would be completely inactivated. Astrocyte Na^+ channels, because of the more favorable membrane potential, would not be inactivated; but the low channel density (see above) would again exclude action potential generation. Of course, the satellite cell Na^+ channels may have no function other than to confuse electrophysiologists; but such speculation is unprofitable. One highly speculative suggestion is that the satellite cells, particularly the Schwann cells, synthesize these channels, which they cannot use, for

later transfer to the neighboring axolemma where they will be used in the process of conduction (Gray and Ritchie, 1985). This local source of sodium channels would supplement (but not replace) the more generally accepted supply by axoplasmic transport from the neuronal cell body in conditions where this transport might be insufficient, especially for the more distal parts of the axolemma, which in man may be as far as one meter from the cell body. Such a neuron would have to supply 500 to 2000 nodes with channels, each with a lifetime of just over 3 days (see Figure 12-2). Apart from the clear economic advantage of such channel transfer, there are at least two pieces of circumstantial evidence in its favor. First, it is known that large molecules the size of proteins can be transferred between axoplasm and



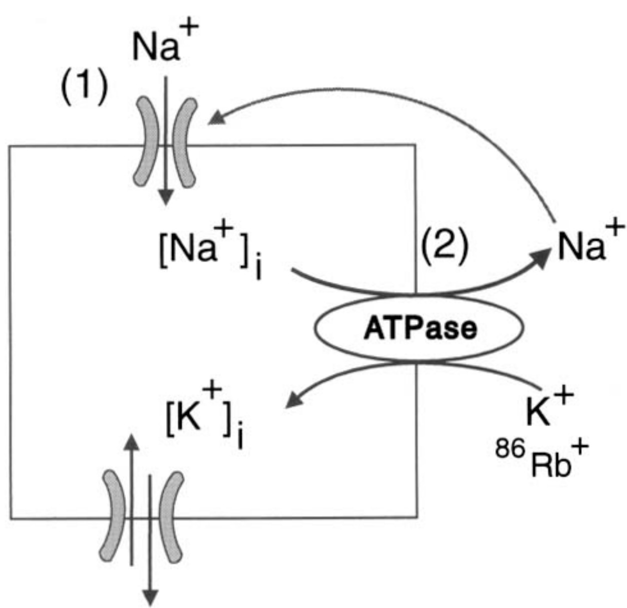


FIG. 12-9. Evidence for function of Na^+ channels as "return" pathway for Na^+/K^+-ATPase. **(A)** Na^+/K^+-ATPase activity was measured as unidirectional influx of $^{86}Rb^+$. $^{86}Rb^+$ uptake determined in spinal cord astrocytes following 15 minutes incubation with the isotope in combination with TTX, *strophanthidin or* TTX, and strophanthidin in combination at the concentrations indicated. (Significant changes in flux were indicated with * and *p*-values plotted next to error bars which represent SEM). Strophanthidin, 100 µM TTX as well as both drugs in combination showed similar reduction in $^{86}Rb^+$ uptake, while 100 nmol TTX did not alter $^{86}Rb^+$ uptake significantly. **(B)** Simplified cell model to explain Na^+ channel-Na^+/K^+-ATPase coupling in spinal cord astrocytes. Na^+ channels function to maintain intracellular Na^+ $[Na^+]_i$ at levels that provide a substrate for Na^+/K^+-ATPase. TTX blocks Na^+ channels (*1*), while strophanthidin and ouabain interfere with activity of the ATPase (*2*).

Schwann cell cytoplasm (Grossfeld, 1991). Although this is different from transfer between Schwann cell plasmalemma and axolemma, at least it is a step in the right direction. Second, there is the morphological finding (Landon and Hall, 1976; Chapter 3, this volume) that the Schwann cell in the nodal region sends a regular hexagonal array of microvilli down onto the axolemma. Although no direct contact between them has yet been shown, the two cell membranes (Schwann cell plasmalemma and axolemma) approach each other much more closely than apposed cell membranes usually do (Landon and Hall, 1976). Indeed, Figure 12-3B clearly shows a section through these microvilli heavily stained with the Na^+ channel antibody. A similar situation applies in the case of astrocytes where Na^+ channel immunoreactivity is observed in astrocyte processes in close apposition to the axolemma at nodes of Ranvier (Black et al., 1989).

Despite its attractiveness, however, the evidence for the transfer theory so far is indirect and speculative. Direct evidence could be provided by molecular biology from a comparison of the molecular structure of the Schwann cell and axonal Na^+ channels—but this is yet to come. It should be pointed out again that from the electrophysiological point of view the Schwann cell Na^+ channels seem to be identical to those in axons (apart from the shift in the *I-V* curve mentioned above). Thus both channels activate with similar kinetics, both have multiple inactivation states, both have similar affinities for tetrodotoxin and saxitoxin. This similarity also extends to potassium channels. Both Schwann cell and axon display an inward rectifier; and both have three types of delayed rectifier channel corresponding with the three types of potassium channel described for frog nerve by Dubois (1981), namely, G_{Kf1}, G_{Kf2}, G_{Ks} (Baker et al., 1993). Again, the molecular identity of the satellite cell and axonal channels remains to be established.

Since astrocyte Na^+ channels are unlikely to be directly engaged in action potential generation, their role is an enigma. It is conceivable, however, that astrocyte Na^+ channels serve roles that do not require regenerative voltage-dependent activation. For at any resting potential a small (but finite) Na^+ permeability must exist, sufficient to allow a small amount of Na^+ ions to leak into the cell (Hille, 1992). Such a Na^+ leak may be important for providing Na^+ ions in high enough concentration for functioning of the astrocyte Na^+/K^+-ATPase, which requires binding of intracellular Na^+ ions to complete its cycle. Indeed, such an involvement of Na^+ channels in the Na^+/K^+-ATPase cycle is known for cultured spinal cord astrocytes (Sontheimer et al., 1994) (Figure 12-9). Thus experiments using the unidirectional influx of $^{86}Rb^+$ as a measure for Na^+/K^+-ATPase activity, show that TTX reduces ATPase activity in a dose-dependent manner. Indeed, at concentrations sufficient for complete Na^+ channel block, TTX is as effective in blocking the Na^+/K^+-ATPase activity as ouabain or strophantidin (Figure 12-9A). This suggests a model (Figure 12-9B) where a Na^+ leak through voltage-activated Na^+ channels maintains the Na^+/K^+-ATPase operational. This is important since one of the main functions of the glial Na^+/K^+-ATPase is believed to be the uptake and removal of extracellular K^+ during spatial buffering. Elevated $[K^+]_0$ would increase the amount of Na^+ ions entering through Na^+ channels since their open probability increases with increasing depolarization. This would stimulate the pump, which in turn would remove extracellular K^+ more rapidly. In this model, therefore, Na^+ channels would facilitate K^+ buffering by the ATPase.

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13 Distribution and transport of chloride and bicarbonate ions across membranes

WOLFGANG WALZ

In the first accounts of glial physiological properties, Cl^- ions were not thought to be involved in the genesis of the membrane potential, or in the function of cellular K^+ homeostasis (Kuffler and Nicholls, 1966). This conclusion was based on the fact that the membrane potential of glial cells showed the same behavior in normal as in Cl^--free salt solution. Additionally, removal of Cl^- ions did not result in any significant membrane potential changes. Over the years, however, more information on the role of Cl^- surfaced (Kimelberg et al., 1979; Kimelberg, 1981; Walz and Schlue, 1982). In addition, with the acceptance of the concept that glial cells not only distribute K^+ passively but also accumulate K^+ and store it transiently, the role of Cl^- became more interesting. The accumulation of K^+ had to be electroneutral and the Na^+ concentration in glial cells was too low to account for a simple 1:1 Na^+:K^+ exchange. It was also found that glial cells could swell rapidly and that Cl^- was involved in this swelling (see Kimelberg and Ransom, 1986; see Chapter 61, this volume). Then Bevan et al. (1985) discovered that under certain circumstances glial cells demonstrated a large permeability for Cl^- ions, stimulating speculations about the functional role of Cl^- channels. Thus, today the question of the role of Cl^- in glial function is a complex one and it looks as if at least two of the three K^+ removal mechanisms involve Cl^- and one also involves HCO_3^- (Walz, 1992). This chapter summarizes the major recent advances in our knowledge about Cl^- and HCO_3^- distribution and transport, as well as function. It should be noted that in the past few years excellent reviews have appeared on this subject (Kettenmann, 1990; Kimelberg, 1990).

DISTRIBUTION

Cerebrospinal fluid in man contains about 119 mM Cl^- (Fishman, 1980), while the Cl^- concentration in rat brain was measured as 134 mM (Smith et al., 1981). Both values are reasonably close to the approximately 140 mM Cl^- that most physiological salt solutions contain. The leech central nervous system, which is directly situated in the blood sinus, does not have a blood-brain barrier. It was found by Zerbst-Boroffka (1970) that leech blood contains only 36 mM Cl^-, with the rest of the anions consisting of high concentrations of lactate, succinate, fumarate, bicarbonate, and citrate. Thus, most artificial salt solutions of researchers working with isolated leech ganglia employ unphysiologically high Cl^- concentrations. Intracellular Cl^- content or concentration were measured by two different methods, radioactive ^{36}Cl flux measurements or the use of Cl^--sensitive microelectrodes. The use of ^{36}Cl is quite straightforward as long as homogeneous monolayer cultures are used, but there are problems in the measurement of cell volume and the calculation of intracellular Cl^- concentration. Almost all researchers working with Cl^--sensitive microelectrodes use the Corning 477315 exchange resin. This charged-carrier exchanger is a quaternary ammonium compound, the precise formulation of which has not been revealed. This exchanger is sensitive to quite a few anions, the most notable is HCO_3^-. Thus, in order to measure the intracellular Cl^- concentration properly, one has to work in nominally HCO_3^--free solutions. These HCO_3^--free conditions are bound to influence the Cl^- gradient due to inhibition of Cl^-/HCO_3^- exchange. A passive Cl^- distribution would result in an intracellular Cl^- concentration of about 5 mM due to the high resting potential. In order to judge if Cl^- is passively distributed [i.e., the chloride equilibrium potential equals the membrane potential ($E_{Cl} = E_M$)] or actively accumulated across a glial cell membrane, the best strategy would be to apply an inhibitor of Cl^- transport (furosemide or bumetanide) and determine if the Cl^- concentration changes to a new value more compatible with passive distribution or if the inhibitor does not change the Cl^- concentration. This approach was used with several preparations (see Table 13-1). In neuropile glial cells of the leech E_{Cl} and E_M are not significantly different, and bumetanide has no effect on the Cl^- concentration (Ballanyi and Schlue, 1990). In cultured oligo-

TABLE 13-1. *Cl^- Distribution and Permeability in Different Glial Cell Preparations*

Preparation	Distribution	Permeability relative to K^+
Leech neuropil glial cells	Passive	High
Glial cells in drone retina	Passive	High
Glial cells in frog spinal cord	Passive	?
Glial cells in olfactory cortex slice	Passive	High
Astrocytes in hippocampal slice	Accumulation	?
Cultured oligodendrocytes	Accumulation by 2–3 mM	Medium
Cultured astrocytes	Accumulation by ~30 mM	Low
Glial cells in cerebral cortex *in vivo*	Accumulation by ~35 mM	?

For references see text.

dendrocytes, measurements with microelectrodes indicated that the intracellular Cl^- concentration was 2 to 3 mM higher than expected if distribution was passive. If the cells were treated with bumetanide their E_{Cl} and E_M were no longer significantly different and the internal concentration decreased by 2 to 3 mM (Hoppe and Kettenmann, 1989a, 1989b). In cultured astrocytes, several reports found ^{36}Cl distributions that are compatible with a 31 to 50 mM intracellular Cl^- concentration (Kimelberg et al., 1979; Kimelberg, 1981; Walz and Hertz, 1983). Application of SITS (4-acetamid-4′-isothiocyanostilbene-2,2′-disulfonic acid) (Kimelberg, 1981) lowers the Cl^- concentration and application of furosemide results in a passive Cl^- distribution (Walz and Hertz, 1983). Measurements with Cl^--sensitive microelectrodes gave a concentration range of 20 to 40 mM in cultured astrocytes (Kettenmann et al., 1987). In this respect, it is of interest that an *in vivo* study (Smith et al., 1981), using rat cerebral cortex, measured the total ^{36}Cl content. The study used the assumptions that the extracellular space is 14% of the total brain volume, that the relative neuron to glial volume is 2:1, and that Cl^- is passively distributed in neurons. It was calculated that the glial intracellular Cl^- concentration of the cortex is between 36 and 46 mM, a result that is close to the measurements of cultured astrocytes, but not of cultured oligodendrocytes. Astrocytes in the hippocampal slice were also found to exhibit active Cl^- accumulation by an indirect method (MacVicar et al., 1989): these cells possess a $GABA_A$ receptor and upon its activation they depolarize. Since this has to involve Cl^- efflux, a nonpassive Cl^- distribution can be assumed. In glial cells of the drone retina, E_{Cl} and E_M are identical (Coles and Orkand, 1984). In the *in vivo* frog spinal cord, unidentified glial cells show a passive distribution of Cl^- (Bührle and Sonnhof, 1983). Unidentified glial cells of olfactory slices have a significantly more positive E_{Cl} than E_M (Grafe and Ballanyi, 1987). However, once the interference of other anions is taken into consideration, the newly corrected E_{Cl} and the E_M are in close correspondence, and one has to assume passive distribution. Unfortunately, the authors did not apply furosemide or bumetanide.

The experiments on Cl^- distribution are summarized in Table 13-1. It is obvious that there is no clear picture about the Cl^- distribution in these different glial preparations. The two invertebrate systems clearly show passive distribution as does the frog spinal cord. Unexplained are the contradictory results from mammalian cortex: *In vivo,* slice and culture preparations show nonpassive distribution for glial cells with the exception of glia in the olfactory cortex slice. However, the Cl^- accumulation of cultured oligodendrocytes is minimal. Such a small accumulation could be easily hidden in the measurements of Ballanyi and coworkers, which would indicate that the unidentified glial cells they were working with could have been oligodendrocytes. It is also quite clear that the Cl^- distribution across astrocytic and oligodendrocytic membranes is very much different. The Cl^- accumulation is accomplished by the Na^+-K^+-Cl^- cotransport system as the inhibitory effect of furosemide and bumetanide indicates. There is also a small contribution by the Cl^-/HCO_3^- exchange transport (Kimelberg, 1981).

The HCO_3^- concentration of human cerebrospinal fluid was measured as 22 mM (Fishman, 1980), which is close to the 26 mM of conventional physiological salt solutions. Intracellular bicarbonate content can be measured in monolayer cultures, since the rapidly equilibrating CO_2 will keep E_{H^+} and $E_{HCO_3^-}$ equal as long as the carbonic anhydrase is not inhib-

ited. Thus one can calculate the intracellular bicarbonate concentration:

$$[HCO_3^-]_i = 10(pH_i - pH_0) \times [HCO_3^-]_0$$

Using this method cultured astrocytes were shown to have an accumulation of 11 mM HCO_3^- and an $E_{HCO_3^-}$ of −27 mV with an extracellular HCO_3^- concentration of 26 mM, which is close to cerebrospinal fluid levels (Walz, 1992).

RESTING PERMEABILITY

The data in Table 13-1, which permit an evaluation of the Cl^- permeability of glial cell membranes, show that those with passive distribution have a high permeability, while those which accumulate intracellular Cl^- have a low permeability. A high Cl^- permeability is usually demonstrated by displacing the membrane potential either by high K^+ or barium application. If there is indeed a high permeability, the E_{Cl} will, within seconds, follow the new E_M. In neuropil glial cells, E_{Cl} follows E_M with a lag of less than 30 seconds (Ballanyi and Schlue, 1990). The same holds true for glial cells of the drone retina (Coles and Orkand, 1984). In unidentified glial cells of the olfactory cortex, E_{Cl} changes with a lag time of less than 10 seconds (Ballanyi et al., 1987). Cultured oligodendrocytes seem to have an intermediate position. If the E_M is changed, E_{Cl} will follow with a lag time of a few minutes (Hoppe and Kettenmann, 1989a, 1989b). In cultured astrocytes, the intracellular Cl^- concentration did not change following displacement of the membrane potential (Kettenmann, 1987). Moreover, ^{36}Cl unidirectional influx analysis of cultured astrocytes showed that 77% of the influx was inhibited by furosemide (an inhibitor of Na^+-K^+-Cl^- cotransport and HCO_3^-/Cl^- exchange), indicating only a small channel-mediated component (Walz and Hertz, 1983).

There is no indication of HCO_3^- conductance in normal glial cells. A previous report (Astion et al., 1987) of a considerable HCO_3^- resting membrane conductance in glial cells of the optic nerve of *Necturus* was reinterpreted by the authors (Astion and Orkand, 1988) as the activity of a Na^+/HCO_3^- cotransport. This transport has a stoichiometry of HCO_3^- to Na^+ that is greater than 1, resulting in HCO_3^- effects on the membrane potential.

VOLTAGE-ACTIVATED ANION CHANNELS

As can be seen from the above discussion, under resting conditions astrocytes in culture have a relatively low Cl^- permeability. There are, however, reports that Cl^- channels can be activated in the cell membranes of astrocytes during certain circumstances. Such a Cl^- conductance was first shown for Schwann cells (Gray et al., 1984). Bevan et al. (1985) were the first to study chloride channels in cultured astrocytes with whole-cell clamp techniques. They found a Cl^- inward flux (outward current) that was activated by potentials of −40 mV or more positive and which did not inactivate. Ascorbate, gluconate, and acetate did not pass through that channel; however, isethionate had a substantial permeability. Unfortunately, the presence of an HCO_3^- permeability was not tested by any of the authors. This indicates an anion channel rather than a specific Cl^- channel. SITS and DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid) inhibited the anion channel. The authors observed too that the whole cell current increased gradually in the 5 to 10 minutes after establishing the seal. Thus, it appeared that intracellular dialysis was removing an inhibitory factor. All these observations are similar to those in the Schwann cells. Other studies (Nowak et al., 1987; Sonnhof, 1987) investigated this large anion conductance of astrocytes at the single-channel level. The single conductance was about 400 to 600 pS. Here it was found that the channel was not noticeable in cell-attached patches, but several minutes after membrane isolation by forming an inside-out patch, the channel openings gradually increased. The existence of these channels in a glial cell *in vivo* preparation was shown by Marrero et al. (1991).

There is some evidence for other Cl^- channels, some of them mediating outward rather than inward currents. Nowak et al. (1987) observed small conductance channels, which were opened by hyperpolarization with 5 pS single-channel conductance. Barres et al. (1988, 1990) found in excised patches from type-1 astrocytes, type-2 astrocytes and oligodendrocytes from the rat optic nerve Cl^- outward currents (Cl^- inward movement) with a single-channel conductance in the range of 25 to 60 pS (see Figure 13-1).

Recently it was shown that cultured rat astrocytes have an anion channel of 200 to 250 pS mediating Cl^- outward movements (Jalonen et al., 1992). The channel is activated by depolarizations, and it is more readily activated when hypoosmotic rather than isosmotic conditions are used. This channel is, like all other single anion channels studied in glial cells so far, only activated in the excised patch. This phenomenon is not yet understood. In their whole-cell clamp studies, Bevan et al. (1985) used pipettes containing ATP, cAMP, and vasoactive intestinal peptide and found no effect on the development of the whole-cell Cl^- current.

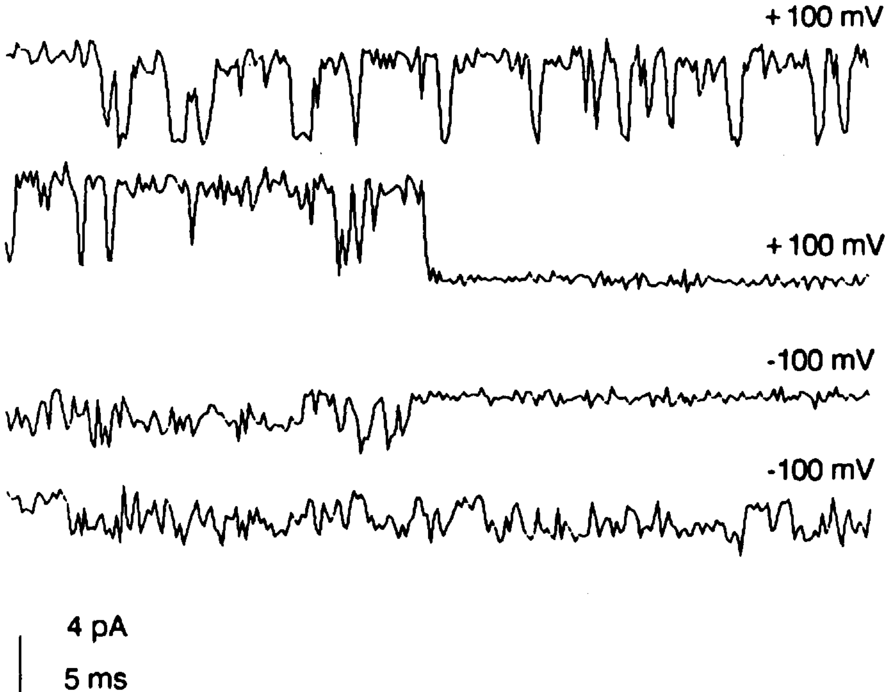

FIG. 13.1 Chloride channel in type-1 astrocytes from rat optic nerve. Single channel recording in an inside-out patch in symmetric TMA-Cl. Channel openings at a holding potential of +100 mV (*top two traces*) have larger amplitudes than those at −100 mV (*lower two traces*). [From Barres et al. (1990), with permission.]

TRANSMITTER-GATED CHLORIDE CHANNELS

α-Adrenergic agonists cause a depolarization in astrocytes (Bowman and Kimelberg, 1988). The authors found that the reversal potential of the response depended on the E_{Cl}; they also were able to change the response by manipulating the extracellular Cl^- concentration. They concluded that α-adrenergic agonists exert their action by opening Cl^- channels.

Gamma-aminobutyric acid (GABA) depolarizes astrocytes (Kettenmann et al., 1987; see also Chapter 21, this volume). The depolarization of astrocytes reverses at the E_{Cl}. The reversal potential is also shifted by changes in the extracellular Cl^- concentration. Patch-clamp recordings from astrocytes (Borman and Kettenmann, 1988) showed that GABA is directly acting on a receptor similar to, but not identical with, the neuronal $GABA_A$ receptor, which is coupled to Cl^- channels. In cultured oligodendrocytes, GABA application triggers an efflux of Cl^-. The intracellular Cl^- concentration falls from 9.2 by 1.3 mM to be close to the E_{Cl}. It appears that this GABA-activated channel is also permeable for HCO_3^-. Kaila et al. (1991) demonstrated that rat astrocytes responded with an acidosis when exposed to muscimol, a $GABA_A$ agonist. This reflects outflux of HCO_3^- through the GABA-activated channel.

Short application of the neurokinin receptor agonist substance P leads to a biphasic depolarization of cultured astrocytes. The rapid and transient depolarization component lasted a few seconds with a reversal potential close to the potassium equilibrium potential (E_K) (Backus et al., 1991). Single-channel recordings increased the opening time of Cl^- channels. It was concluded that activation of neurokinin receptors in astrocytes modulates the activity of K^+ and Cl^- channels, leading to a complex depolarization of the membrane potential.

POSSIBLE FUNCTION OF CHANNEL-MEDIATED CHLORIDE FLUXES

Boyle-Conway Mediated Potassium Chloride Uptake

Glial cells are involved in K^+ clearance of the extracellular space (Walz, 1989; see Chapter 47, this volume). A spatial buffer mechanism is in all likelihood operating in cells that have a high and selective K^+ permeability. As can be seen from Table 13-1, there are at least three types of glial cells that have a high resting Cl^- permeability. Walz (1982) was the first to suggest (based on the high Cl^- permeability of leech neuropile glial cells) that these cells can take up KCl passively if the extracellular K^+ is increased. This would involve channel-mediated KCl inward movements until $E_K = E_{Cl}$. Since that time, intracellular K^+ and Cl^- measurements showed that an extracellular K^+ increase results in KCl movements into the glial cells, presumably mediated passively through channels. In glial cells of the drone retina (Coles et al., 1986), glial cells from the olfactory cortex slice (Ballanyi et

al., 1987), neuropile glial cells of the leech (Wuttke, 1990), and cultured oligodendrocytes (Hoppe and Kettenmann, 1989a), it was shown that the main response to physiological stimuli or small extracellular K^+ increases was an increase in the concentration of KCl in glial cells. These four preparations are the only ones to show a high or medium Cl^- resting permeability. This suggests that one main function of this Cl^- permeability is the mediation of passive KCl increase during neuronal activity. One result of this function would be a concomitant cell swelling.

Such a Boyle-Conway-mediated KCl uptake could also play a role in glial cells with no measureable resting Cl^- permeability. In this case a large depolarization would be needed to open the large anion conductance channel (see Figure 13-2). To reach the −40 mV of the membrane potential at which these channels are usually activated one would have to increase the extracellular K^+ concentration to approximately 35 mM, a concentration that occurs only in pathophysiological events like hypoxia-ischemia, spreading depression, and trauma. Another problem is the mechanism that keeps these channels normally closed. One would have to postulate an intracellular regulatory mechanism that would remove this block. Bevan et al. (1985) were the first to suggest such a mechanism. There is some experimental evidence for this. If astrocytes in culture are exposed to 60 mM K^+, their K^+ increases by 45 mM. The Cl^- concentration increases by 20 mM and the HCO_3^- concentration by 22 mM (Walz and Mukerji, 1988; Walz, 1992). If Cl^- is replaced by gluconate, the K^+ increase is inhibited by 50%, and if isethionate (which has a high permeability for the anion channel) is used as substituent, the K^+ accumulation rates are not affected. Thus, the authors postulated a Boyle-Conway mediated K^+, Cl^- and HCO_3^- increase accompanied by a 40% volume increase. The pathophysiological implications of this mechanism are obvious, although one has to point out that a 40% swelling is only observed in cell cultures, whereas *in vivo* the extent of the swelling will be much reduced.

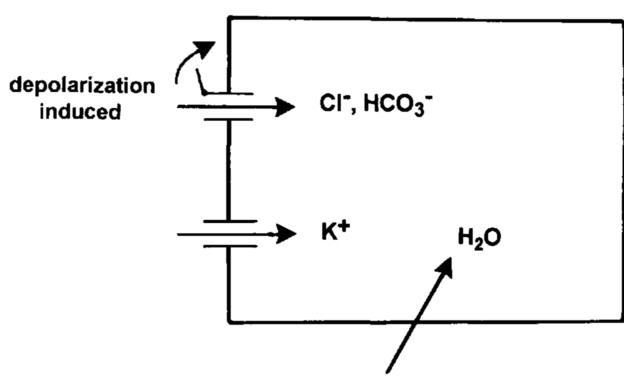

FIG. 13-2. Scheme of a Boyle-Conway mediated anion and K^+ accumulation involving the large anion conductance.

Neurotransmitter-Evoked Release of Potassium Chloride

Bormann and Kettenmann (1988) postulated a model of Cl^- regulation by glial cells at GABAergic synapses. The activation of postsynaptic neuronal GABA receptor channels induces an inwardly directed movement of Cl^-. Glial cells in the vicinity of the synaptic cleft react with efflux of Cl^-, which could buffer the loss of Cl^- from the extracellular space. The physiological significance of this buffering is, however, questionable. With a Cl^- concentration of about 140 mM in the extracellular space, the relative Cl^- changes are small and no processes sensitive to such changes are known.

Other authors (Bowman and Kimelberg, 1988) favor the concept that KCl release from astrocytes exposed to GABA and glutamate would serve to influence neuronal excitability. Walz (1982) postulated that a transmitter-evoked release of KCl from leech neuropil glial cells could serve as the return signal for glial cells to release temporarily stored K^+ back into the extracellular space for its eventual return into neurons.

CHLORIDE-BICARBONATE EXCHANGE AND SODIUM-BICARBONATE COTRANSPORT

These two anion transport mechanism are closely involved in internal and external pH regulation (see Figure 13-3). Kimelberg et al. (1979) and Kimelberg

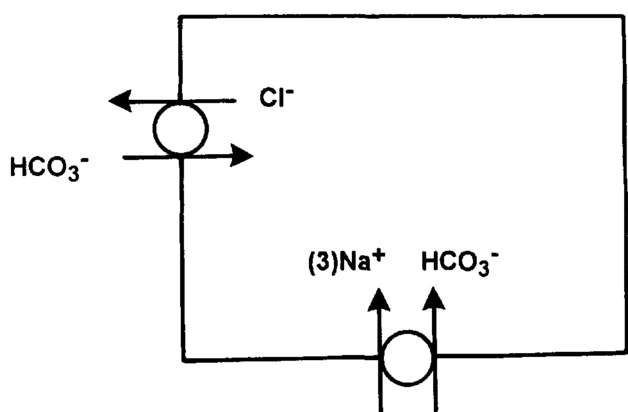

FIG. 13-3. Scheme of carriers involving HCO_3^- transport.

(1981) presented evidence for a SITS-inhibitable Cl^-/HCO_3^- and Cl^-/Cl^- exchange. The Cl^-/Cl^- exchange might be due to the inhibition of Cl^- channels by SITS (Bevan et al., 1985) and might therefore be nonexistent. However, the evidence for the Cl^-/HCO_3^- exchange was convincing. Measurements on leech neuropile glial cells (Deitmer and Schlue, 1987, 1989) demonstrated that this Cl^-/HCO_3^- exchange was a Na^+-dependent Cl^-/HCO_3^- exchange, with a steep dependency on intracellular pH. The exchange mechanism is electroneutral with the energy being provided by the Na^+ gradient (Thomas, 1984). Kettenmann and Schlue (1988) failed to find evidence for this exchange mechanism in cultured oligodendrocytes. This exchange mechanism couples Cl^- and pH regulation.

A Cl^--independent pH regulation is provided by the Na^+-HCO_3^- cotransporter, which is also SITS and DIDS sensitive (except in cultured oligodendrocytes) (Kettenmann and Schlue, 1988). Deitmer and Schlue (1989) found evidence for such an electrogenic Na^+-HCO_3^- cotransport in leech glial cells (either 1:2 or 1:3 coupling ratio Na^+:HCO_3^-). The same was found for glial cells in the mudpuppy optic nerve (Astion and Orkand, 1988). In cultured oligodendrocytes this cotransport seems to be electrically neutral (Kettenmann and Schlue, 1988). The electrogenic nature of the transporter makes its action dependent upon the membrane potential. A depolarization will stimulate its turnover rate. Deitmer (1991) found in leech glial cells that the cotransport can reverse its action and lead to HCO_3^- secretion from the cells (see Chapter 14, this volume).

SODIUM-POTASSIUM-CHLORIDE COTRANSPORT

The first direct evidence for an electroneutral, coupled transport of Na^+, K^+, and Cl^- together, came from work by Geck et al. (1980) on mouse tumor ascites cells. The authors described net fluxes of Na^+, K^+, and Cl^-, which were furosemide-sensitive. The ratio between the fluxes did not vary with the concentration of the ions, and the movement of any one ion required the presence of the other two. Moreover, the fluxes were unaffected by the membrane potential and vice versa. Accordingly, Geck et al. (1980) proposed an electroneutral triple cotransport of Na^+, K^+, and Cl^- in the proportions 1:1:2, respectively. Since 1980, this cotransport was demonstrated, in many tissues, and is reputed to play a role in functions such as epithelial absorption, secretion, cell volume regulation, and intracellular Cl^- accumulation.

Kinetic Properties

The existence of a furosemide-sensitive Cl^- and K^+ transport in astrocytes was first described by Kimelberg (1979). A detailed study of the kinetics of the carrier in cultured rat astroyctes was made by Tas et al. (1986, 1987). These authors found that about 30% of the unidirectional K^+ flux was sensitive to furosemide during steady-state conditions. With the use of Hill plots for Na^+, K^+, and Cl^- the authors found the Hill coefficients for Na^+, K^+, and Cl^- to be 1.06, 0.99, and 1.90, respectively. These values are close to the theoretically predicted stoichiometry of 1:1:2, confirming the action of a true Na^+, K^+, and Cl^- carrier and not another form of a furosemide-sensitive transport. The authors also found that only Br^- could replace Cl^- and that gluconate and acetate were ineffective, in agreement with reported properties of the carrier in other systems. An analysis of the activity of the Na^+, K^+, and Cl^- cotransporter on the extracellular concentrations of these ions gave the following $K_{0.5}$ values: the half-maximal activation concentration for K^+ was 2.7 mM; the one for Na^+ was 35 mM; and the one for Cl^- was 40 mM. It is of special interest to point out that the $K_{0.5}$ is almost identical with the K^+ concentration (3 mM) in the cerebrospinal fluid. Thus, small increases of extracellular K^+ would have a maximal stimulatory effect on the carrier, resulting in K^+ uptake into astrocytes.

Involvement in Volume Control

The involvement of the carrier in volume changes of rat astrocytes was investigated by Kimelberg and Frangakis (1985). Increasing the osmolarity of the medium from 320 to 520 mOsm by addition of mannitol resulted in shrinkage of the cells. Maximum shrinkage occurred within 5 minutes, reaching a new steady state of 0.78 μl/mg protein from an initial value of 1.96. The same treatment had no effect on the Cl^- content. Thus, the cells appear to be behaving as osmometers, responding with marked shrinkage to exposure to hypertonic medium due, presumably, to loss of water. The presence of 1 mM furosemide had no significant effect. Exposure of the cells to hypotonic medium resulted in cell swelling. This was followed by considerable recovery of volume (regulatory volume decrease). Furosemide did not affect the volume recovery. After reexposure to isotonic medium, the cells shrank rapidly and then began to return to their original volume. This restoration of volume was partially inhibited by furosemide. There was also an initial rapid loss of K^+ in hypotonic medium. This was then followed by reuptake of K^+, when the cells were subsequently ex-

posed to isotonic medium. The reuptake of K^+ after exposure to isotonic medium was partially inhibited by furosemide, similar to its effects on the recovery of the water space under the same conditions. Thus, there is compelling evidence that the $Na^+/K^+/Cl^-$ carrier is involved in volume regulation in astrocytes. However, this happens only during the secondary volume recovery after previous cell shrinkage due to exposure to isotonic medium, in cells which have already been exposed by hypotonic medium.

Active Chloride Accumulation

Walz and Hertz (1983) measured the Cl^- distribution in mouse astrocytes. It was found that the Cl^- content at steady state was 158 nmol/mg protein or about 25 to 30 mM. Since the membrane potential is about −75 to −80 mV, one has to conclude that Cl^- is not passively distributed but actively accumulated in astrocytes in such a way that its equilibrium potential is about −40 mV. When the cells are treated with furosemide, the K^+ content and the membrane potential were unaffected. However, the cells lost Cl^- resulting in a new Cl^- content of 68 nmol/mg protein. This amounts to a concentration of about 10 to 12 mM. This new concentration would be compatible with a equilibrium distribution of Cl^-, and with a Cl^- equilibrium potential that is similar to the membrane potential. Thus, one can conclude that the furosemide-sensitive carrier is responsible for active accumulation of Cl^- in astrocytes. Hoppe and Kettenmann (1989a) found similar results for cultured oligodendrocytes: Bumetanide resulted in a passive distribution of Cl^-.

Involvement in Potassium Accumulation

The involvement of a $Na^+/K^+/Cl^-$ carrier in the clearance of excess K^+ in the extracellular space was investigated by Walz and Hinks (1985, 1986) and Walz and Mukerji (1988). The studies focused on changes in the net content of K^+ in mouse astrocytes rather than the unidirectional K^+ influx. The cells were equilibrated at 3 mM K^+ and then exposed to 12 mM K^+. The cells increased their K^+ content by 390 nmol/mg protein or 63% within 50 s following the increase of external K^+. Ouabain, the Na^+-K^+ adenosine triphosphatase (ATPase) inhibitor, inhibited 240 nmol/mg of this net uptake. The same amount, 240 nmol/mg, was inhibited by furosemide application alone. Obviously, there is an overlap of the amount inhibited by ouabain and furosemide. Part of the uptake by one transport system is inhibited by the inhibition of the other system. A similar observation was made by Tas et al. (1986) for unidirectional K^+ fluxes of mouse astrocytes.

The furosemide-sensitive part of the net K^+ uptake was absent during Cl^- removal and reduced to about one-third in low-Na^+ solutions. This is a clear sign that furosemide-sensitive K^+ uptake is coupled to Cl^- and Na^+ fluxes; a mechanism which is similar to the one in rat astrocytes.

The water content of the mouse astrocytes increased by 27% during the 50 seconds following an increase of extracellular K^+ from 3 to 12 mM. Furosemide treatment alone did not change the water content of the astrocytes. However, in furosemide-treated cells that were exposed to 12 mM K^+, there was no swelling. Exposure to 12 mM K^+ in Cl^--free conditions did not produce a significant swelling.

In another set of experiments, the Na^+ gradient of the cells was changed by preincubation of the cells with zero K^+ solution. This treatement leads to a loss of intracellular K^+ and its replacement by Na^+ ions. In this way, the normal Na^+ content of the mouse astrocytes, about 200 nmol/mg protein, could be raised to 900 nmol/mg. Thus, the inward driving force for Na^+ could be abolished. If such cells, which were preincubated in zero K^+ and were now Na^+ loaded, were exposed to 12 mM K^+, there was a considerable net uptake of K^+ (400 nmol/mg) within the 1st min of exposure to 12 mM K^+. However, furosemide treatment had no effect on this net uptake, suggesting that a furosemide-sensitive or $Na^+/K^+/Cl^-$-mediated transport component does not contribute to this K^+ uptake, since the main driving force, the Na^+ inward gradient, was abolished. Thus, apart from the presence of Cl^- ions, the carrier needs an inward Na^+ driving force to function properly in its role during K^+ net uptake.

If the external K^+ was raised from 12 to 60 mM K^+, a net K^+ uptake of about 700 nmol/mg protein occurred within 1 minute. This amounts to a doubling of the K^+ content. The furosemide-sensitive component of this net uptake was minimal: 50 nmol/mg. However, the major part of this net uptake at these high, pathological K^+ concentrations was still inhibited by Cl^- removal, but only if Cl^- was replaced by glucuronate and not if replaced by isethionate. This clearly signals the participation of large conductance anion channels, which are permeable to many anions and which are activated only by large depolarizations. The driving force of the Na^+ gradient during the exposure to 60 mM K^+, when the cells depolarize from −85 to −10 mV, is greatly reduced. Thus, it is not surprising that under these conditions the inward movement of ions via the $Na^+/K^+/Cl^-$ carrier is largely dysfunctional and

replaced by passive Boyle and Conway mediated forces.

A Transmembrane Sodium Cycle

The previous observations, namely, a Cl^- and Na^+ dependence of the furosemide-sensitive K^+ net uptake, the overlap of the ouabain- and furosemide-sensitive components, and the dependency on an inward driving force for Na^+, all can be explained by a model of interdependency of the $Na^+/K^+/Cl^-$ carrier with the Na^+/K^+-ATPase. In this model, a coupling of both carriers via the intracellular Na^+ concentration results in a recycling of the Na^+ ion across the membrane. The cycle is illustrated in Figure 13-4. It involves a Na^+/K^+-ATPase that is stimulated by a high external K^+ and high intracellular Na^+. Elevated external K^+ also stimulates the $Na^+/K^+/Cl^-$ carrier, transporting these ions inward, since the sum of all driving forces will point inward. As a result, Na^+ is cycled across the membrane, carried inward by the $Na^+/K^+/Cl^-$ carrier and returned by the Na^+/K^+-ATPase. The whole mechanism leads to intracellular KCl accumulation and inward movement of water to compensate for increased osmolarity. At higher, probably pathological, K^+ levels, the driving force for the $Na^+/K^+/Cl^-$ carrier will be small. Consequently, this mechanism will lose importance as external K^+ levels progress beyond 10 mM K^+.

OUTLOOK

The results and concepts presented in this chapter are based on the assumption that intracellular Cl^- and HCO_3^- are evenly distributed in the cells. Recently, Hara et al. (1992) used cultured rat hippocampal neurones with a Cl^--sensitive fluorescent dye. They found an uneven distribution in the following order: processess >> periphery > central region. They attributed this to the localized distribution of a Cl^- pump and the Na^+-K^+-Cl^- cotransport. Kimelberg and Bourke (1982) first speculated on asymmetric arrangements of different transport mechanisms of the astrocytic perineuronal and perivascular membranes. This would serve to secrete HCO_3^- into the perineuronal space and H^+ into the perivascular region. It is of interest that Newman (1991) localized the Na^+/HCO_3^- cotransporter in freshly dissociated Müller cells from salamander retina and found a preferential localization in the endfeet regions. One can expect that the future use of fluorescent dyes will bring new findings and concepts regarding asymmetric arrangements of transport processes that will revolutionize our present view of anion transport.

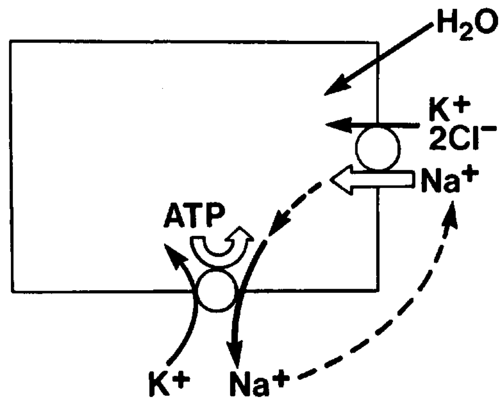

FIG. 13-4. Scheme of a transmembrane Na^+ cycle. For explanation see text.

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14 pH regulation

JOACHIM W. DEITMER

pH regulation in nervous systems is an essential homeostatic function of the cellular elements known as glial cells and neurons. This is mainly achieved by transport of acid-base equivalents across the cell membranes. Substances transported out of these cells may be accumulated in the narrow spaces between nerve and glial cells—the extracellular or interstitial spaces—the third compartment in nervous tissue besides the two cellular elements (see Chapter 26, this volume).

The regulation of pH by glial cells in nervous systems raises two fundamental questions: (1) What are the mechanisms of intraglial pH regulation; and (2) Are these mechanisms involved in controlling the pH in the extracellular space (ECS)? In order to evaluate the role of glial cells in the overall H^+ homeostasis in nervous tissue, the production of acid-base equivalents by neurons, and the pH changes in the extracellular space, must be taken into account. These are discussed here only insofar as they may affect intraglial pH; the reader is therefore referred to some excellent reviews of these issues (Roos and Boron, 1981; Nattie, 1983; Moody, 1984; Thomas, 1984; Nicholson et al., 1985; Chesler, 1990; Chesler and Kaila, 1992; Ranson, 1992).

pH changes in nervous systems may have some important consequences for glial cell functions. As discussed in other chapters of this volume, intraglial acidification may uncouple glial cells and change their input resistance electrically (Tang et al., 1985; Walz and Wuttke, 1989; Kettenmann et al., 1990; see Chapter 19, this volume) and may lead to cell swelling during lactacidosis (Kraig and Petito, 1989; Staub et al., 1990; see Chapter 61, this volume) or to other pathophysiological changes (Hansen, 1985; Siesjö, 1988; Kraig and Chesler, 1990; see Chapter 64, this volume).

ELECTROCHEMICAL H^+ GRADIENT

Most living cells studied so far maintain an alkaline intracellular pH (pH_i) with respect to the H^+ electrochemical equilibrium. Therefore, cells must *actively* control their pH_i, which means that acid equivalents must be extruded out of the cells against a H^+/OH^- electrochemical gradient. Since the production of H^+ exceeds the H^+ consumption in most metabolic processes (Hochachka and Mommsen, 1983), cells and tissues have to extrude acid constantly. In addition, any leak of acid-base equivalents across the cell membrane would lead to a flow of H^+ into, or OH^- and HCO_3^- out of, the cell down their electrochemical gradient, which must be compensated by active transport in the opposite direction.

The H^+ equilibrium potential, at which H^+/OH^- and HCO_3^- are passively distributed across the cell membrane, is given by the Nernst equation:

$$E_H = \frac{RT}{F} * \ln \frac{[H^+]_0}{[H^+]_i}, \qquad (1)$$

where $[H^+]_0$ and $[H^+]_i$ are the concentrations of intra- and extracellular H^+ concentration, R is the gas constant, T the absolute temperature, and F the Faraday constant. This can be rewritten as

$$E_H = 58.5 \text{ mV } (pH_i - pH_0), \qquad (2)$$

where pH_i is the intracellular, and pH_0 the external pH; T was assumed to be 295 K (22°C). Equation (2) predicts that at a pH_0 of 7.4 (40 nM H^+) and a pH_i of 7.1 (80 nM H^+), the distribution of H^+ achieves an equilibrium at a cell membrane potential of −18 mV. This is far more positive than the membrane voltage of most cells. Consequently, at potentials more negative than −18 mV, H^+ must be actively extruded, although the intracellular H^+ concentration is twice that of the external fluid. In the example given, H^+ efflux has to overcome an electrochemical gradient of 57 mV, equivalent to about 1 pH unit.

If a membrane potential of −75 mV is assumed, as is more typical for glial cells, a passive H^+ distribution at pH_0 7.4 would give a pH_i value of 6.1 (coincidentally the pK′ of carbonic acid). This, however, is far too acid for most living cells to survive for any length of time due to inhibition of major enzymes at this low pH. Hence active pH_i regulation in glial cells is essential and can be predicted from these theoretical considerations alone.

pH IN THE GLIAL MICROENVIRONMENT

The constant extrusion of acid equivalents out of the cells results in some accumulation of acid in the extracellular spaces, rendering it 0.1 to 0.3 pH units more acid than the blood, cerebrospinal fluid, or bulk solution, both in vertebrates (e.g., Cragg et al., 1977; Urbanics et al., 1978; Javaheri et al., 1983) and invertebrates (Voipio et al., 1991; Deitmer, 1992a; Rose and Deitmer, 1992). These acid equivalents can be transported as H^+/OH^- or HCO_3^-, or as carboxylic acids, such as lactic acid (Kraig et al., 1985; Nicholson et al., 1985; Walz and Mukerji, 1988). The more acid microenvironment also reduces the H^+ electrochemical gradient across the cell membranes by 0.1 to 0.3 pH units, corresponding to 6 to 18 mV.

A remarkable exception has recently been observed in the stomatogastric ganglion of the crab (*Crustacea*), where the pH in the extracellular spaces (pH_e) was more *alkaline* than in the bulk solution (Golowasch and Deitmer, 1993). It appears as though additional mechanisms of pH_e control operate in this ganglion known for its coordinated, rhythmical, neuronal activity (Selverston and Moulins, 1987).

The steady-state pH_e and the pH_e shifts are affected by inhibition of the carbonic anhydrase, as, for example, with acetazolamide or ethoxyzolamide (Thomas et al., 1991; Chen and Chesler, 1992; Deitmer, 1992a; Kaila et al., 1992; Rose, 1993). Glial cells have been reported to contain a major fraction of the carbonic anhydrase in nervous tissue (Giacobini, 1962; Sapirstein et al., 1984), although, from the changes in experimentally induced pH_e shifts produced by carbonic anhydrase inhibition, it is suggested that either there is a considerable amount of carbonic anhydrase also in the extracellular space (Chen and Chesler, 1992; Deitmer, 1992b; Kaila et al., 1992) or carbonic anhydrase is located in the cell membranes with access to both intra- and extracellular compartments (Wistrand, 1984).

Acid and alkaline transients of up to several tenths of a pH unit can be recorded in the extracellular space during neuronal activity (Kraig et al., 1983; Somjen, 1984; Endres et al., 1986; Krishtal et al., 1987; Ranson et al., 1987; Chesler and Chan, 1988; Chvatal et al., 1988; Jarolimek et al., 1989; Walz, 1989; Sykova and Svoboda, 1990; Jendelova and Sykova, 1991; Rose and Deitmer, 1992; Rose, 1993) or under pathophysiological conditions, such as anoxia or ischemia (Thorn and Heitmann, 1954; Crowell and Kaufmann, 1961; Kraig et al., 1985; Krnjevic and Walz, 1989; Katsura et al., 1992; Ransom and Philbin, 1992; Ransom et al., 1992), spreading depression (Mutsch and Hansen, 1984; Chesler and Kraig, 1989; Scheller et al., 1992), or epileptiform activity (Siesjö et al., 1985; Takizawa et al., 1991; Laxer et al., 1992; Martins da Silva and Deitmer, 1993). In the ischemic brain, lactic acid levels may rise dramatically, leading to a severe acidification in the extracellular space; this lactacidosis may lead to glial cell swelling, impairment of synaptic transmission, or even cell death (Lomneth et al., 1990; Staub et al., 1990; Walz and Harold, 1990; Nedergaard et al., 1991; Scheller et al., 1992). It has been suggested that glial cells may help to control pH_e by mechanisms that tend to damp large pH_e transients, and thereby play an important role in maintaining H^+ homeostasis in nervous tissue (Siesjö, 1985; Ransom et al., 1987; Woodbury et al., 1988; Jendelova and Sykova, 1991; Kaila et al., 1991; Deitmer, 1992a; Ransom, 1992; Rose and Deitmer, 1994). In a recent study it was suggested that the pH_i regulation of astrocytes derived from *jimpy* mice, which carry a genetic disorder of myelination, is indeed abnormal, and the steady-state pH_i is considerably higher than in normal astrocyte cultures (Knapp et al., 1993).

pH_e changes may well be physiologically important. The conductance and/or gating of many membrane channels and carriers may be modulated by pH_e. This has been shown for γ-aminobutyric acid (GABA) receptor channels (Kaila et al., 1990), for the *N*-methyl-D-aspartate (NMDA)-type glutamate receptor channel (Tang et al., 1990; Traynelis and Cull-Candy, 1990), and for Ca^{2+} channels (Konnerth et al., 1987; Jarolimek et al., 1990; Kovalchuk et al., 1990; Mironov and Lux, 1991). This, in turn, may have severe consequences for neuronal excitability and information processing in the nervous system.

There is also evidence that pH_e changes not only modify neuronal ion channels, but also channels in the glial membrane. In glial cells of an oligodendrocyte lineage a rapid decrease of pH_0 elicited large Na^+ currents (Sontheimer et al., 1989). In cultured Schwann cells (Hoppe et al., 1989) and in leech neuropile glial cells (Munsch and Deitmer, 1991), the K^+ conductance was affected by pH.

MEASUREMENTS OF INTRAGLIAL pH

Direct intracellular pH measurements in glial cells were started only a few years ago (Chesler and Kraig, 1987; Deitmer and Schlue, 1987), much later than in neurons (Thomas, 1974; Boron and DeWeer, 1976), which have been studied extensively with respect to their pH regulation for about 20 years. This is not only due to the lack of interest in glial cells as

compared to nerve cells for a long time, but also due to the difficulties identifying cells as *glial* cells within nervous tissue, and to record from these glial cells directly. Apart from the rising interest in glial cells in the last 10 years, new techniques for identifying glial cells and measuring intracellular pH have led to substantial progress in this field.

The most commonly used technique for measuring intraglial as well as interstitial pH has been pH-sensitive, neutral carrier microelectrodes (Ammann et al., 1981), which impale the cell and provide an "on-line" recording of pH_i. The development of ion-sensitive fluorescent dyes (Heiple and Taylor, 1982; Rink et al., 1982), has created another powerful method to monitor pH_i directly and continuously. While the use of pH-sensitive microelectrodes is an "invasive" technique, since the electrode has to impale the cell, pH-sensitive fluorescent dyes, such as BCECF (2',7'-(biscarboxyethyl)-5,6-carboxyfluorescein), can be loaded via their membrane-permeable acetoxymethyl ester (AM), which is cleaved off in the cell by intrinsic diesterases. In addition, pH-sensitive microelectrodes give point measurements of pH_i and are, due to their very high electrical input resistance, slow as compared to the fluorescent dyes, which are fast and can provide spatial information of intracellular H^+ distribution.

On the other hand, in a tissue, the acetoxymethyl ester derivative of the fluorescent dyes would unselectively diffuse into different types of cells, unless injected by a conventional or patch-clamp microelectrode into individual cells, which would render this method "invasive" as well. Then, using fluorescent dyes, the absolute pH_i values can only be determined by H^+/K^+-permeabilizing the cells at the end of an experiment (e.g., using nigericin). This may be less accurate than determining pH_i with a pH-sensitive microelectrode, and only reveals the absolute pH_i value *after* an experiment. Hence roughly speaking, pH-sensitive microelectrodes are preferable in cells $\geq$ 15 to 20 μm in intact tissue, while fluorescent dyes are likely to be used in very small cells and in cell cultures.

Other techniques to measure pH_i, such as the distribution of the weak acid DMO (5,5'-dimethyl-2,4-oxazolidine) (Waddell and Butler, 1959; Chow et al., 1991, 1992), or the phosphorus shift in nuclear magnetic resonance (NMR) (Moon and Richards, 1973; Laxer et al., 1992) are either very costly (NMR), or may be less direct and/or reliable.

pH_i has been determined in a variety of glial cell types. The steady-state pH_i values obtained in different glial cells, either in intact tissue or in culture, ranged between 6.68 and 7.6 (Table 14-1). In all

TABLE 14-1. *Steady-State pH_i Values and Intrinsic Buffering Power (β_i) in Different Glial Cell Preparations in HEPES- and/or in CO_2/HCO_3^--Buffered Salines*

Preparation	T(°C)	Steady-State pH_i Buffer Used: HEPES	CO_2/HCO_3^-	$[CO_2]$, %	pH_o	β_i at Steady-State pHi (in mM)	Method of Measurement	Authors
Rat cortical astrocytes *in vivo*	35–37		7.10	5	7.3		pH-sensitive microelectrodes	Chesler and Kraig, 1987
Rat cortical astrocytes *in vivo*	37		7.04	5	7.41		pH-sensitive microelectrodes	Chesler and Kraig, 1989
Neuropile glial cells of intact leech ganglia	22–25	6.87	7.18	2	7.4	20–30	pH-sensitive microelectrodes	Deitmer and Schlue, 1987
Neuropile glial cells of intact leech ganglia	22–25	6.97	7.25	5	7.4		pH-sensitive microelectrodes	Deitmer, 1991
Connective glial cells of the leech	22–25	7.09	7.37	2	7.4		pH-sensitive microelectrodes	Szatkowski and Schlue, 1992
Cultured mouse oligodendrocytes		7.2	7.6	2	7.4		pH-sensitive microelectrodes	Kettenmann and Schlue, 1988
Cultured mouse astrocytes		6.68	7.05	5	7.35		pH-sensitive microelectrodes	Wuttke and Walz, 1990
Astrocytes in optic nerve of *Necturus*	20–30	7.32	7.39	2.3	7.5		pH-sensitive microelectrodes	Astion et al., 1991
Cultured mouse astrocytes	37	6.97	7.16	2	7.4	37.7	[14C]DMO distribution	Chow et al., 1991
Cultured rat astrocytes	33–34	7.02	7.22	5	7.4	20	Fluorimetry BCECF-AM	Kaila et al., 1991
Cultured rat astrocytes	37	6.89	7.24	5	7.4		Fluorimetry BCECF-AM	Boyarski et al., 1993

DMSO, 5,5'-dimethyl-2,4-oxazolidine; BCECF, 2',7'-(biscarboxyethyl)-5,6-carboxyfluorescein; AM, acetoxymethyl ester.

glial cells, where pH_i was measured in different buffer systems, pH_i was lower in HEPES-buffered salines (6.68 to 7.32; mean 7.04 ± 0.2) than in CO_2/HCO_3^- buffered salines (7.04 to 7.6; mean 7.22 ± 0.16), corresponding to an average H^+ concentration in glial cells of 91 nM and 60 nM, respectively. The H^+ equilibrium potentials calculated from these values and an extracellular pH of 7.4 are −21 mV and −11 mV, respectively (at room temperature). This indicates the importance of CO_2/HCO_3^- for the maintenance of the alkaline steady-state pH_i.

INTRAGLIAL BUFFERING POWER

The intrinsic, intraglial buffering power (β_i) has only been determined in some studies; it varied between 20 and 37.7 mM (Table 14-1). The value of β_i includes the cytoplasmic, physicochemical buffering as well as the organellar buffering power, measured as the "instantaneous" change in pH_i as response to the addition of strong acid or base, in the absence of CO_2/HCO_3^- (for a more elaborate description of buffering, see Roos and Boron, 1981; Boron, 1985; Szatkowski and Thomas, 1989).

The total intracellular buffering power (β_T) is the sum of the intrinsic buffering power, β_i, and the CO_2/HCO_3^--dependent buffering power, β_{CO_2}. The latter is negligible in the nominal absence of CO_2/HCO_3^-, but may add considerable intracellular buffer capacity in the presence of 2 to 5% CO_2/10 to 26 mM HCO_3^-. β_{CO_2} depends upon the concentration of CO_2 and pH_i, which determines the intracellular HCO_3^- concentration. The capacity of intracellular H^+ buffering by CO_2/HCO_3^- is matched by the amount of intracellular HCO_3^- used, and can be written as

$$\beta_{CO_2} = \frac{\Delta[HCO_3^-]_i}{\Delta pH_i}, \quad (3)$$

where $\Delta[HCO_3^-]_i$ is the change in intracellular HCO_3^- concentration, and $\Delta\ pH_i$ the change in pH_i. The $[HCO_3^-]_i$ can be calculated by the modified Henderson-Hasselbalch equation

$$[HCO_3^-]_i = 10^{(pH_i - pH_o)} * [HCO_3^-]_o, \quad (4)$$

where $[HCO_3^-]_o$ is the external HCO_3^- concentration. Thus, equation (3) yields in first approximation

$$\beta_{CO_2} = 2.3\ [HCO_3^-]_i. \quad (5)$$

For example, from a value of 7.2 for pH_i, measured in a saline buffered with 5% CO_2/24 mM HCO_3^- at pH 7.4, the $[HCO_3^-]_i$ can be calculated to be 15.1 mM, which adds 34.8 mM buffering power to the cytoplasm. These are realistic values for glial cells, as well as for neurons and many other cell types; thus, the presence of CO_2/HCO_3^- can easily double or triple the buffering capacity provided by the intrinsic buffering power. In a cell with a steady-state pH_i of 7.2 in a CO_2/HCO_3^--buffered saline, the total buffering power may hence add up to 60 mM (assuming a β_i of 25 mM, and a β_{CO_2} of 35 mM, see above).

A pH_i value of 7.2 corresponds to a free H^+ concentration of 63 nM, and a buffering power of 60 mM predicts that the addition of 60 mM strong acid or base will change the pH by one unit. The ratio of free to "buffered" H^+ ($r_{H^+/H}$) is hence given by

$$r_{H^+/H} = \frac{10\ [H^+]_i}{\beta_T} \quad (6)$$

This means that, with the figures given above, for each free H^+ there are approximately 10^5 bound acid/base equivalents. If a cell is loaded with a large amount of acid, it will use most of its buffer capacity, so that a subsequent, equally large acid load will change pH_i much more. This is due to the fact that most H^+ binding sites were occupied by the first acid load. Consequently, the buffering power is greatly dependent on the actual pH_i value and upon the pK's of the intracellular buffering sites. Hence intracellular buffering is rapid and efficient on short term, but once saturated, it becomes inefficient.

Another way to cope with an acid load is to sequester acid equivalents into intracellular organelles, such as mitochondria and lysosomes ("organellar buffering power"). In most studies, this type of H^+ sequestering was not separated from the purely physicochemical buffering, which is why both are usually combined to the *intrinsic* buffering power. It is yet unclear, what role intracellular H^+ sequestration plays in controlling cytoplasmic pH in glial cells, but, in the long run, this organellar buffering power would approach saturation, unless acid equivalents are extruded from cells to maintain the cellular H^+ household.

Since the outward transport of acid equivalents or inward transport of base equivalents must be driven against the H^+/OH^- gradient in glial cells, as in most other cell types, they require some form of energy. These transport forms constitute the *active pH_i regulation* via membrane pumps and carriers.

MECHANISMS OF ACTIVE pH_i REGULATION

Like many other cell types, glial cells seem to use transport systems for their pH_i regulation. With most cells they share the use of a Na^+/H^+ exchanger and a Cl^-/HCO_3^- exchanger; with most *epithelial* cells they share a Na^+/HCO_3^- cotransporter, which

is absent in neurons and most nonepithelial cells. Recently, a bafilomycin-sensitive H^+ pump has been suggested in hippocampal astrocytes (Pappas and Ransom, 1993).

Little is known about the role of second messengers (see Chapter 34, this volume) on pH_i regulation in glial cells. The steady-state pH_i and the pH_i changes in leech glial cells were unaffected by intracellular Ca^{2+} transients evoked by membrane depolarization (Deitmer et al., 1993), suggesting that the homeostasis of H^+ and Ca^{2+} is not necessarily interrelated in these glial cells. There is evidence that neurotransmitters or hormones affect intraglial pH directly (Deitmer and Munsch, 1992) or by changing the glial membrane potential (see below) (Deitmer and Szatkowski, 1990) which may be mediated by intracellular messengers, such as cyclic nucleotides (J. W. Deitmer, unpublished observations). However, further studies are needed to elucidate the role of intracellular second messengers as modulators of intraglial pH.

Sodium/Hydrogen and Chloride/Bicarbonate Exchangers

Evidence for a Na^+/H^+ exchanger has been found in various glial cell types, as in cultured astrocytes (Kimelberg et al., 1979; Chow et al., 1991; Mellergard and Siesjö, 1991; Boyarski et al., 1993; Shrode and Putnam, 1994), in leech neuropile and connective glial cells (Deitmer and Schlue, 1987; Szatkowski and Schlue, 1992), in optic nerve astrocytes (Astion et al., 1989), in cultured oligodendrocytes (Kettenmann and Schlue, 1988), in rat Schwann cells (Nakhoul et al., 1994), and in glial or glioma cell lines (Benos and Sapirstein, 1983; Jean et al., 1986; Jacubovicz et al., 1987; Shrode and Putnam, 1994). The main criteria for the presence of a Na^+/H^+ exchanger, which drives H^+ out of the cells by using the energy of the Na^+ gradient with a stoichiometry of 1:1, are the following: (1) pH_i recovery from an acid load occurs in CO_2/HCO_3^--free saline; (2) pH_i recovery is dependent on external Na^+ and a large inwardly directed Na^+ gradient, and (3) independent of anions, such as Cl^- and HCO_3^-; and (4) the sensitivity of pH_i recovery to the K^+-sparing diuretic amiloride (Benos, 1982).

Cl^-/HCO_3^- exchange across glial cell membranes, either Na^+-independent or Na^+-dependent, has been reported for cultured astrocytes (Bourke et al., 1978; Kimelberg et al., 1979; Kimelberg, 1981), for cultured oligodendrocytes (Kettenmann and Schlue, 1988), and for leech glial cells (Deitmer and Schlue, 1987; Szatkowski and Schlue, 1992). This exchanger is identified by its dependence on Cl^- and HCO_3^-, and can be inhibited by SITS and/or DIDS, stilbene derivatives, which are known to inhibit anion carriers in cell membranes (Cabantchik and Rothstein, 1972). It may be inferred by analogy from studies on other cell types that the Na^+-independent Cl^-/HCO_3^- exchanger primarily *extrudes* HCO_3^-, and thereby helps to recover pH_i following an alkaline load (Thomas, 1977; Vaughan-Jones, 1979; Simchowitz and Roos, 1985).

The evidence for a Na^+-dependent Cl^-/HCO_3^- exchanger is based on pH_i recovery from an acid load, which shows similar properties as the Na^+-*independent* Cl^-/HCO_3^- exchanger, with the difference that it is dependent on the presence of external Na^+ and that it usually transports base equivalents *into* the cells. However, it is uncertain, whether Na^+ is actually transported by this carrier, and further characterization of this transport mechanism is needed to evaluate its role in intraglial pH regulation.

Both classical transporters for pH_i regulation, the Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger, although clearly identified in glial cells, have not been investigated in as great detail in glial cells as in some other cell types (see Wieth et al., 1982; Grinstein and Rothstein, 1986). This may also be the result of the apparent dominance of the Na^+-HCO_3^- cotransporter for intraglial pH regulation as found in a variety of glial cells. The impact of the presence of CO_2/HCO_3^- on pH_i regulation from an acid load in leech neuropile glial cells is shown in Figure 14-1. Recordings from different cells using the same protocol are shown, one with a double-barreled pH-sensitive microelectrode (Figure 14-1A), and one using the wavelength ratio 440 nm:495 nm of a cell filled with the fluorescent pH-sensitive dye BCECF (Figure 14-1B). The maximum rate of recovery from an intracellular acidification induced by an ammonium prepulse (see Roos and Boron, 1981) was five to ten times greater in the presence of 5% CO_2/24 mM HCO_3^- than in the nominal absence of CO_2/HCO_3^-. This is largely due to activation of an inwardly directed Na^+-HCO_3^- cotransport in these cells by the addition of CO_2/HCO_3^-, which also hyperpolarized the glial membrane (Figure 14-1) due to the electrogenic nature of the Na^+-HCO_3^- cotransporter.

Sodium-Bicarbonate Cotransporter

First evidence for a Na^+-HCO_3^- cotransporter in glial cells was found in the giant neuropile glial cells of the leech (Deitmer and Schlue, 1987, 1989), in astrocytes in the optic nerve of the mudpuppy *Necturus* (Astion and Orkand, 1988; Astion et al., 1989)

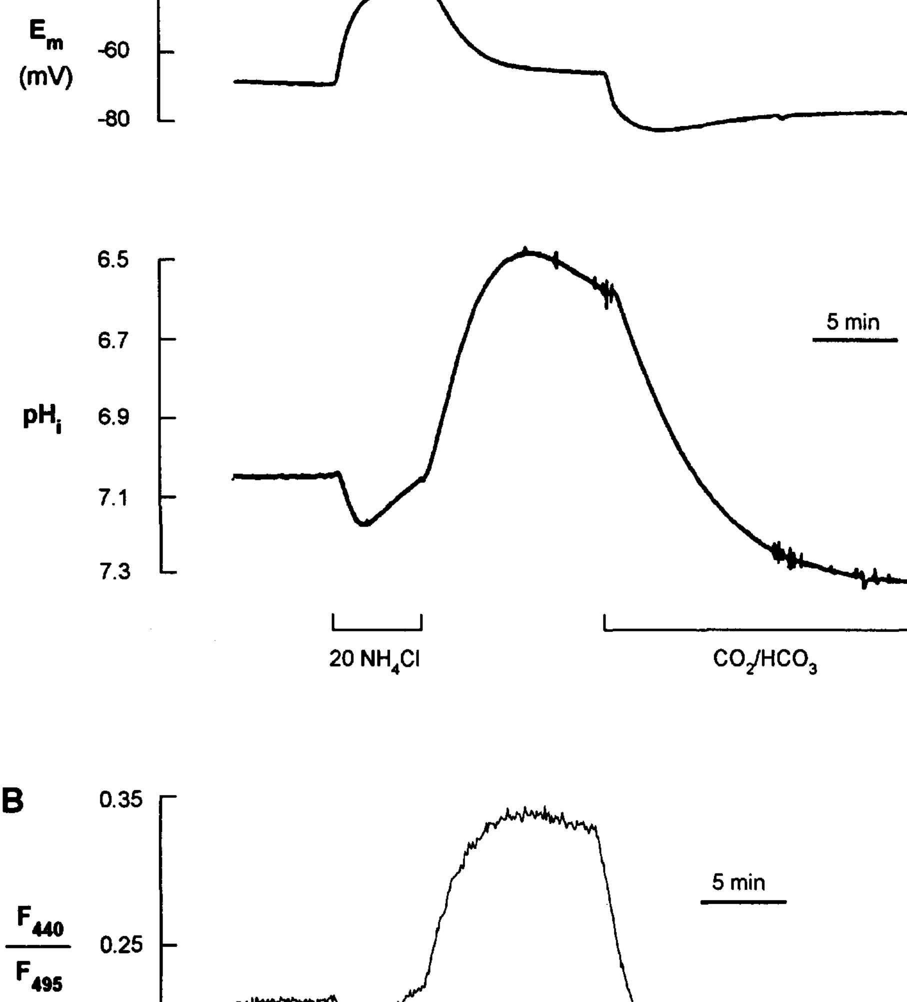
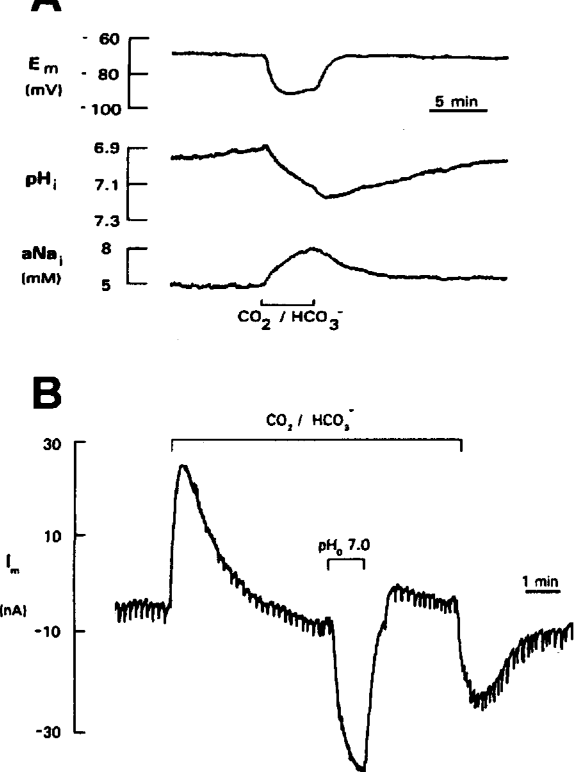


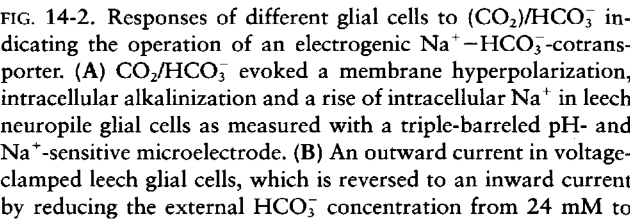
FIG. 14-1. (A) Intracellular recording of membrane potential (E_m, *upper trace*) and pH (pH_i, *lower trace*) in a leech neuropile glial cell using a double-barreled pH-sensitive microelectrode. After the application of ammonium (20 NH_4 Cl), the cell acidified. The recovery of pH_i in CO_2/HCO_3^--free, HEPES-buffered saline was slow, its maximum rate was 0.025 pH units/min. Following the addition of 5% CO_2/24 mM HCO_3^-, the pH_i recovery accelerated considerably; its maximum rate increased to 0.125 pH units/min. Note that the steady-state pH_i leveled at a more alkaline value in the presence of CO_2/HCO_3^-. (B) The same protocol was carried out in another neuropile glial cell using BCECF to measure intracellular pH, indicated by the fluorescence ratio 440 nm:495 nm (F440/F495), showing an even more dramatic change in pH_i recovery following the addition of CO_2/HCO_3^- [Figure B: from W. Nett and J. W. Deitmer, unpublished data.]

and in cultured mouse oligodendrocytes (Kettenmann and Schlue, 1988). In addition, a Na^+-HCO_3^- cotransporter was found in freshly dissociated Müller glial cells of the salamander retina (Newman, 1991; Newman and Astion, 1991), in cultured mouse astrocytes (Chow et al., 1991), and in leech connective glial cells (Szatkowski and Schlue, 1992). We have recently obtained evidence for an electrogenic Na^+-HCO_3^- cotransport in rat cerebellar astrocytes in culture, measuring a Cl^--independent and HCO_3^--dependent change in pH_i, and a Na^+- and HCO_3^--dependent current (Brune et al., 1994); similar results were reported for primary rat astrocytes (Shrode and Putnam, 1994).

In most of these studies, the Na^+-HCO_3^- cotransporter was detected during addition or removal of CO_2/HCO_3^-; addition of CO_2/HCO_3^- resulted in an intracellular alkaline shift, while removal of CO_2/HCO_3^- reversed this pH_i change (Figure 14-2A). Simultaneously, the glial membrane hyperpolarized and depolarized, respectively, during these buffer changes.

Since the CO_2/HCO_3^--dependent pH_i shifts and membrane potential changes were dependent upon the presence of external Na^+, but not on external or intracellular Cl^-, it was concluded that the underlying process might be an electrogenic Na^+-HCO_3^- cotransporter, similar in nature to that first described in proximal tubule cells of the salamander kidney (Boron and Boulpaep, 1983), and then in





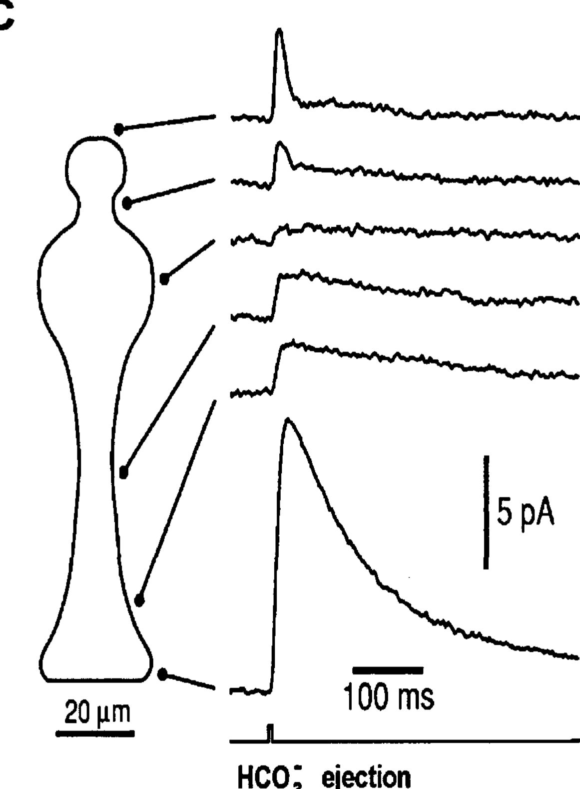


FIG. 14-2. Responses of different glial cells to $(CO_2)/HCO_3^-$ indicating the operation of an electrogenic $Na^+-HCO_3^-$-cotransporter. (A) CO_2/HCO_3^- evoked a membrane hyperpolarization, intracellular alkalinization and a rise of intracellular Na^+ in leech neuropile glial cells as measured with a triple-barreled pH- and Na^+-sensitive microelectrode. (B) An outward current in voltage-clamped leech glial cells, which is reversed to an inward current by reducing the external HCO_3^- concentration from 24 mM to 10 mM (pH_o 7.0) or by removal of CO_2/HCO_3^-. (C) Ejection of HCO_3^- from a pipette onto different regions of a salamander retinal Müller glial cell elicited outward currents of different amplitude, suggesting inhomogeneous distribution of electrogenic $Na^+-HCO_3^-$ cotransporter sites with preferential localization at the glial cell endfoot. [Figures A and B: from J. W. Deitmer, T. Munsch, M. Reusch, and H. P. Schneider, unpublished data; Figure C: from Newman (1991), with permission.]

many other epithelial cells (for references see Boron and Boulpaep, 1989). Indeed, there was a reversible rise in intracellular Na^+ during the exposure to CO_2/HCO_3^- (Figure 14-2A; see also Deitmer and Schlue, 1989; Deitmer, 1992b). In the absence of external Na^+, addition of CO_2/HCO_3^- neither produced the intracellular alkalinization nor a membrane hyperpolarization or outward current in leech glial cells (Munsch and Deitmer, 1994) and in rat astrocytes (Brune et al., 1994), indicating that there is no detectable HCO_3^- conductance in these glial cells.

In voltage-clamp, the addition of CO_2/HCO_3^- produced an outward current; subsequent reduction of the external pH by reducing the HCO_3^- concentration evoked an inward current (Figure 14-2B), consistent with an inward going electrogenic Na^+-HCO_3^- cotransport upon addition of CO_2/HCO_3^-, and a reversed, outward going cotransporter upon reduction of pH_o (and $[HCO_3^-]_o$) (Deitmer, 1991; Munsch and Deitmer, 1994; Brune et al., 1994).

As in epithelial cells, the Na^+-HCO_3^- cotransporter in glial cells could be inhibited by stilbene derivatives, such as DIDS, SITS, or DNDS, but was unaffected by amiloride. In retinal Müller glia, the DNDS-sensitive, Na^+- and HCO_3^--dependent current showed an inhomogeneous distribution (Newman, 1991) (Figure 14-2C), suggesting a higher density of Na^+-HCO_3^- cotransporter molecules in the glia endfoot than in the rest of the cell membrane. It could be shown in retinal glial cells that this HCO_3^--dependent current was directly dependent on the Na^+ gradient (Newman, 1991). It will be interesting to elucidate the function of this glial Na^+-HCO_3^- cotransporter in the retina, in particular as this cotransporter has also been found in retinal pigment epithelium (Hughes et al., 1989; La Cour, 1989).

The rate of pH_i change in leech glial cells was directly dependent on the extra- and intracellular HCO_3^- concentrations (Deitmer, 1991). The cotransporter could be reversed in leech glial cells (Deitmer and Szatkowski, 1990; Deitmer, 1991; Munsch and Deitmer, 1994) and in retinal Müller glial cells

(Newman, 1991; Newman and Astion, 1991), that is, operating inwardly *and* outwardly, depending on the thermodynamic conditions (Figure 14-2A, B). The stoichiometry reported for these two types of glial cells, however, was different. A stoichiometry of $2HCO_3^-$:$1Na^+$ was determined from the Na^+ and HCO_3^- gradients across the cell membrane of leech neuropile glial cells, as measured with ion-sensitive microelectrodes (Deitmer and Schlue, 1989; Deitmer, 1992b) and from the current reversal potential of −74 mV in a two-microelectrode voltage clamp (Munsch and Deitmer, 1994). In retinal Müller glial cells a stoichiometry of $3HCO_3^-$:$1Na^+$ was measured by means of the current reversal potential near −25 mV using the whole-cell patch-clamp technique. Since the stoichiometry is of crucial importance for the functional significance of such an electrogenic Na^+-HCO_3^- cotransporter, because it determines the *direction* of the transport, it should be considered in more detail.

With the intracellular Na^+ and HCO_3^- (pH) measured, the equilibrium potential (or reversal potential) of the Na^+-HCO_3^- cotransporter, E_{NaHCO_3}, is given by (see Boron and Boulpaep, 1983; Deitmer and Schlue, 1989)

$$E_{NaHCO_3} = \frac{RT}{(n-1)\cdot F} \ln \frac{[Na^+]_o\,[HCO_3^-]_o^n}{[Na^+]_i\,[HCO_3^-]_i^n}, \qquad (7)$$

where n is the HCO_3^-:Na^+ stoichiometry, and R, T, and F have their usual meanings. Assuming a stoichiometry of $n = 2$, the E_{NaHCO_3}, where no net transport is mediated by the carrier, was −74 mV in leech neuropile glial cells, at pH_i of 7.25 (17 mM HCO_3^-) and pH_o of 7.4 (5% CO_2/24 mM HCO_3^-), 10 mM free intracellular Na^+ concentration and 90 mM external Na^+ (Deitmer, 1992b). This corresponds well with the membrane resting potential (−73 mV) and the reversal of the Na^+-, HCO_3^--dependent current in voltage clamp (−74 mV) (Munsch and Deitmer, 1994). If a stoichiometry of $n = 3$ is assumed, E_{NaHCO_3} would be −41 mV under these conditions, which means that the cotransporter would extrude Na^+-HCO_3^- at potentials negative to −41 mV, and stimulation of the cotransporter would normally *depolarize* the cell membrane, until the membrane potential equals the equilibrium potential of the Na^+-HCO_3^- cotransporter. Measurements of membrane potential changes, voltage-clamp currents, and intracellular ion activities, however, clearly indicates a stoichiometry of $n = 2$.

This stoichiometry implies that the cotransporter operates near its equilibrium at normal resting membrane potential. Depolarization stimulates inward-going, and hyperpolarization stimulates outward-going Na^+-HCO_3^- cotransport accompanied by intracellular alkalinization and acidification, and by outward and inward currents, respectively (Figure 14-3A), rendering intraglial pH and acid-base flux across the glial membrane very sensitive to membrane potential changes (Deitmer and Szatkowski, 1990; Munsch and Deitmer, 1994; Deitmer and Schneider, 1995). The maximal rates of pH_i change, converted to base (HCO_3^-) flux and the peak currents were directly and similarly correlated with membrane voltage (Figure 14-3B).

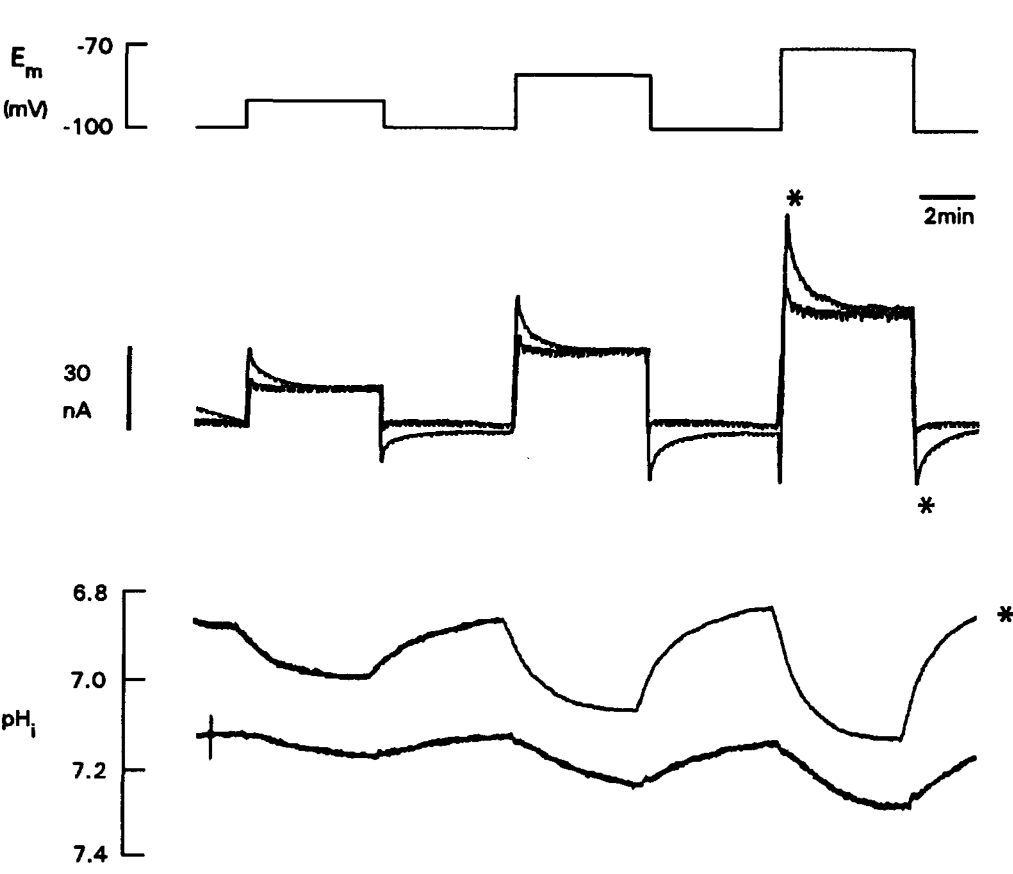
The reversal potential of the cotransporter in retinal Müller glial cells, determined in whole-cell clamp using the HCO_3^--dependent, DNDS-sensitive current, gave a stoichiometry of 3 HCO_3^-:1 Na^+ (Newman, 1991; Newman and Astion, 1991), as found in most epithelial cells (Boron and Boulpaep, 1989). This suggests that, at rest, a sustained outwardly directed cotransporter operates in these cells, acidifying the cytosol, and providing base equivalents to the retinal extracellular space.

With a stoichiometry of $1Na^+$:$3HCO_3^-$, the outwardly directed HCO_3^- gradient and the negative membrane potential would overcome the large inwardly directed Na^+ gradient and extrude Na^+ and HCO_3^-. Hence stimulation of the cotransporter would acidify the cytoplasm and depolarize the cell membrane. Only large depolarizations of the cell membrane well beyond −40 mV (−25 mV) would reverse the cotransport to an inwardly directed mode.

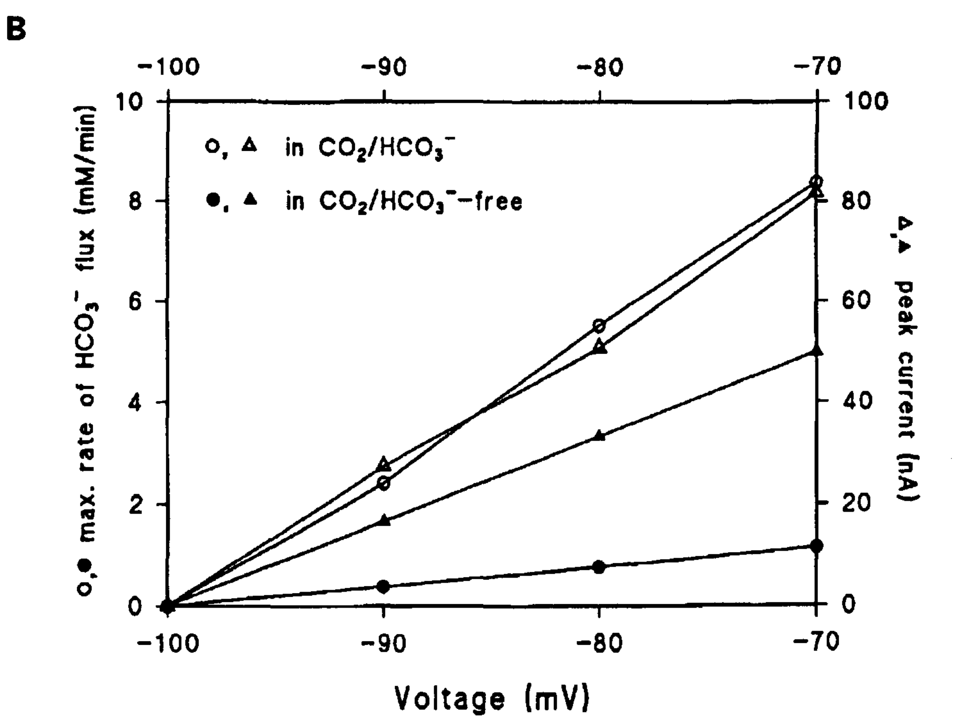
In an electroneutral cotransport, Na^+ and HCO_3^- would be carried into the cell due to the large inwardly directed Na^+ gradient, which is about twice that of the HCO_3^- outward gradient. An electroneutral Na^+-HCO_3^- was reported for cultured oligodendrocytes (Kettenmann and Schlue, 1988), where it thus would function as a mechanism to *increase* pH_i.

EFFECT OF NEURONAL ACTIVITY AND NEUROTRANSMITTERS ON INTRAGLIAL pH

It is of great interest, how far and by what mechanisms the steady-state pH_i and the pH_i regulating processes in glial cells are affected by electrical activity of, and neurotransmitter release by, neurons. In concert with other ions and metabolites, H^+ changes in glial cells, neurons and in the ECS may be part of the neuronal-glial cross talk.



A
E_m
(mV)
-70
-100
2min
30
nA
*
*
6.8
7.0
7.2
7.4
pH_i
*



B
-100
-90
-80
-70
10
8
6
4
2
0
100
80
60
40
20
0
o, △ in CO_2/HCO_3^-
●, ▲ in CO_2/HCO_3^--free
o,● max. rate of HCO_3^- flux (mM/min)
△,▲ peak current (nA)
Voltage (mV)

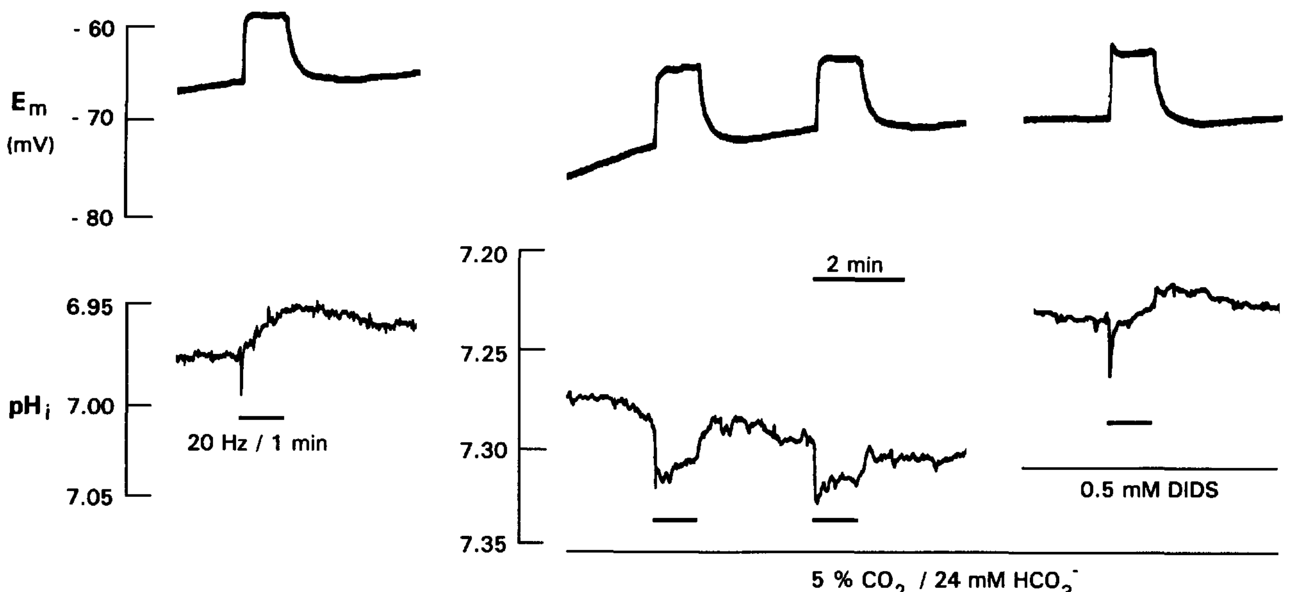


FIG. 14-4. Stimulation-induced changes in membrane potential (E_m, *upper traces*) and pH (pH_i, *lower traces*) of a leech neuropile glial cell in nominal CO_2/HCO_3^--free, HEPES-buffered saline (left panel) and in the presence of CO_2/HCO_3^- (*middle and right panels*). The stimulation-induced alkalinization in CO_2/HCO_3^--buffered saline was reversed by DIDS (diisothiocyanatostilbene-2,2′-disulfonic acid), which blocks the $Na^+-HCO_3^-$ cotransporter in this cell. Stimulation occurred by applying a voltage to a side nerve of a leech segmental ganglion via suction electrode. [From Rose (1993), with permission.]

Neuronal Stimulation

Stimulation of the cortical surface of rat brain (20 Hz) produced a rapid, sustained membrane depolarization (20 mV) and an intracellular alkalinization of about 0.2 pH units in astrocytes in the frontal cortex (Chesler and Kraig, 1987, 1989). In leech neuropile glial cells stimulation of a side nerve caused a similar depolarization, and a rapid intracellular alkalinization, which was substantially reduced by DIDS or in the absence of CO_2/HCO_3^- (Figure 14-4) (Rose and Deitmer, 1992; Rose, 1993). It has been suggested that the glial depolarization is presumably due to the accumulation of extracellular K^+, released by the neurons during activity, while the depolarization might be the cause for the intracellular alkalinization. This membrane depolarization would stimulate inwardly directed, electrogenic Na^+-HCO_3^- cotransport and hence alkalinize the glial cytoplasm. This DIDS-sensitive uptake of base equivalents (Figure 14-4) significantly reduces the stimulation-induced alkaline transient in the extracellular space (Rose, 1993; Rose and Deitmer, 1994) and supports the hypothesis that glial acid-base regulation helps to control extracellular pH (Deitmer, 1992a; Ransom, 1992).

At the end of the stimulation, the glial membrane repolarized, which was accompanied by a recovery of pH_i. Sometimes, the membrane slightly hyperpolarized, and the pH_i decreased transiently. This is in accordance with the dependence of pH_i on the membrane potential due to the activity of the electrogenic Na^+-HCO_3^- cotransporter in the glial membrane (Deitmer and Szatkowski, 1990; Munsch and Deitmer, 1994; Deitmer and Schneider, 1995). Membrane hyperpolarization would drive the cotransporter in the outward direction, acidifying the glial cytoplasm and providing base equivalents to the ECS (Deitmer, 1991, 1992a; Rose and Deitmer, 1994).

Action of Neurotransmitters

Glial cells have a large number of neurotransmitter receptors, which might be, similar to neurons, cou-

FIG. 14-3. (A) Recording of intracellular pH (*lower traces*) and membrane current (*middle traces, superimposed*) during slow 5-minute voltage steps between −70 and −100 mV (*upper trace*) in the absence and in the presence (*) of $CO_2-HCO_3^-$. Note the additional, largely transient, extra currents and the increased pH_i changes in the saline containing 5% CO_2−24 mM HCO_3^- (B) Maximum rate of acid-base flux (*left*) and peak current (*right*) plotted versus the membrane voltage during 5-minute voltage-clamp steps (for the experiment shown in Figure A) in the presence (○, △) and absence (●, ▲) of 5% CO_2−24 mM HCO_3^-. [From Munsch and Deitmer (1944), with permission.]

pled to ion channels (see Chapters 21 and 22, this volume). Transmitters and their agonists may lead to changes of intraglial pH, for example, due to some permeability of these channels to H^+/OH^- or HCO_3^-. Therefore, intraglial pH changes observed during neuronal stimulation may also be due to the action of neurotransmitters, which are released during neuronal stimulation. However, so far intraglial *alkalinizations* have mainly been reported during neuronal stimulation (see above), and intraglial *acidifications* following the action of neurotransmitters.

A fall of pH_i in cultured rat astrocytes was measured following the activation of $GABA_A$ receptors (Kaila et al., 1991). Since $GABA_A$ receptor channels are not only permeable to Cl^-, but also to HCO_3^- (Kaila and Voipio, 1987; Voipio et al., 1991), HCO_3^- moves down its electrochemical gradient through these channels out of the cell, thereby acidifying the cell interior, and alkalinizing the extracellular space (Chen and Chesler, 1992; Kaila et al., 1992). Indeed, a fall in pH_i mediated by $GABA_A$ receptor channels was monitored also in rat astrocytes (Kaila et al., 1991), and in leech neuropile glial cells (J. W. Deitmer, unpublished observations). The pH_o changes were affected by $GABA_A$ receptor blockers and by inhibition of carbonic anhydrase, which catalyzes the dissociation of carbonic acid. Local ejection of GABA into the CA1 region of rat hippocampus caused a HCO_3^--dependent, picrotoxin-sensitive, rapid extracellular alkaline shift, similar as can be observed during cortical stimulation (Chen and Chesler, 1990, 1992). Interestingly, the stimulation-induced extracellular alkaline transient was blocked by picrotoxin, an inhibitor of $GABA_A$ receptor channels, in rat hippocampal slices (Kaila et al., 1992), but significantly increased in the leech central nervous system (Rose, 1993).

Glutamate and acetylcholine cause an acid shift in glial cells (Figure 14-5). Activation of the kainate-type glutamate receptor produced a fall of 0.2–0.3 pH units in leech glial cells (Deitmer and Munsch, 1992), suggesting that this receptor channel is not only permeable to Na^+ and Ca^{2+} (Deitmer and Munsch, 1992; Müller et al., 1992), but also to H^+. Figure 14-5A shows the membrane potential change and intracellular acidification of a leech neuropile glial cell upon application of the glutamate agonist kainate. This acidification was not dependent upon HCO_3^-, and the cell recovered from this acidosis within minutes. Carbachol, an agonist of acetylcholine receptors, elicited a smaller membrane depolarization and an intracellular acidification of 0.1 to 0.2 pH units (Figure 14-5B).

The intraglial acidification produced by acetylcholine, and its agonists carbachol and nicotine, ranged between 0.09 and 0.33 pH units in CO_2/HCO_3^--free saline, and could be reduced by external Zn^{2+} (2 mM) (Ballanyi and Schlue, 1989; Schneider, 1990). Again, H^+ permeation through acetylcholine-gated channels in the glial membrane would be the simplest explanation for this pH_i decrease, although this has to be confirmed by further studies. In the presence of CO_2/HCO_3^- a multiphasic pH_i was evoked by carbachol, whereby the intracellular acidification was followed and partly masked by an alkalinization (Schneider, 1990; J. W. Deitmer, unpublished observations). The latter may be due to the concomittant membrane depolarization, stimulating inward-going Na^+-HCO_3^- cotransport.

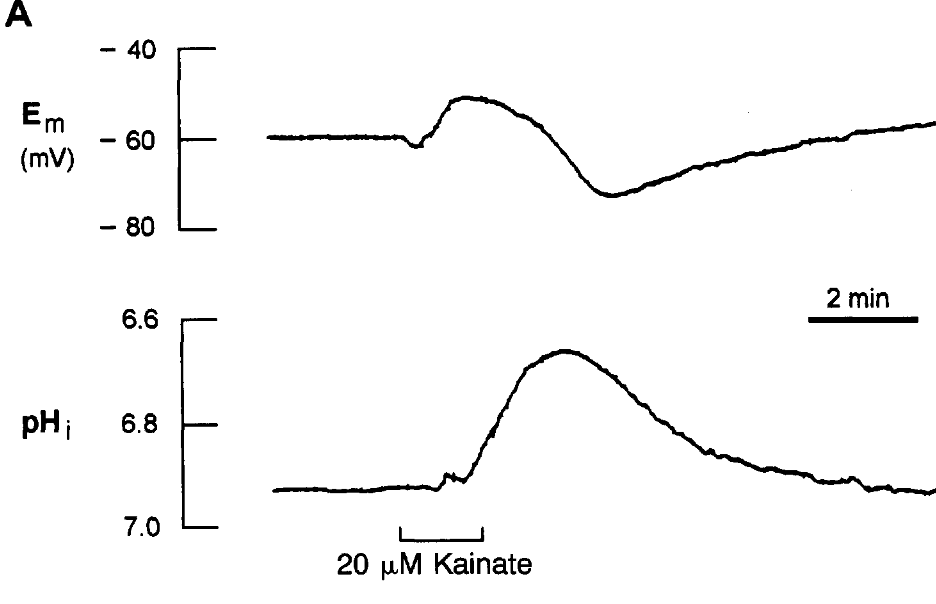
Local ejection of glutamate into the hippocampus produced an extracellular alkaline shift, similar to that following stimulation of the Schaffer collaterals (Chen and Chesler, 1992). While inhibition of carbonic anhydrase abolished the GABA-induced extracellular alkaline shift, it enhanced the alkaline shifts evoked by glutamate and Schaffer collaterals, indicating that the latter arises through a HCO_3^--independent mechanism, while the GABA-evoked alkalinization is consistent with a HCO_3^- efflux through $GABA_A$-receptor channels.

In the leech central nervous system, kainate and carbachol induced biphasic pH shifts in the extracellular space, that is, a brief alkalinization followed by a large acidification, while GABA and serotonin (5-HT) had little or no effect on the extracellular pH (Rose, 1993).

Although the impact of neurotransmitter-induced intraglial pH changes have only begun to be understood, it becomes clear that these processes may well take part in the modulation of pH in the extracellular space, particularly in the vicinity of synapses, where transmitters may transiently accumulate. This may be of special functional significance, since the conductance and/or gating of neurotransmitter- and voltage-activated neuronal and glial channels can be directly modulated by H^+ (see Chapter 2).

CONCLUSIONS

Since the first intracellular pH measurements in glial cells *in vivo* only some years ago, it has become clear now that glial cells possess a variety of processes that modulate intraglial pH as well as the pH in the extracellular space. Figure 14-6 summarizes the main membrane processes relevant for perturbing and regulating intraglial pH. The largest pH_i changes appear to be contributed by the neurotransmitters and the reversible, electrogenic Na^+-HCO_3^- cotrans-



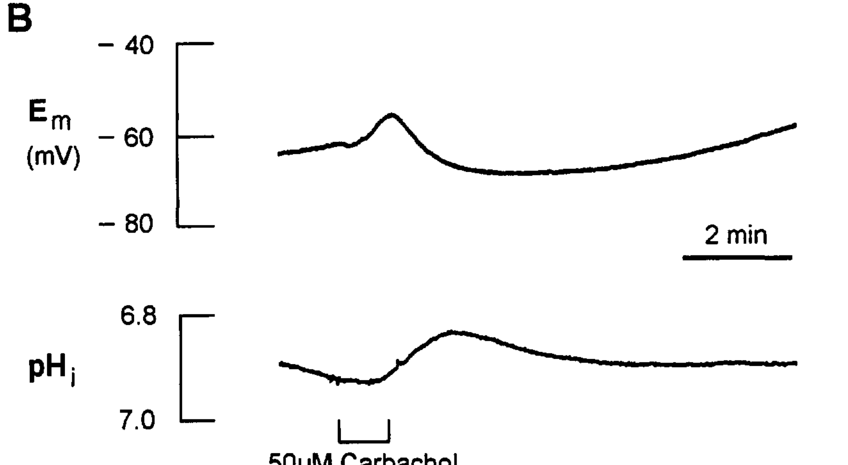


FIG. 14-5. (A) The effect of the glutamate receptor agonist kainate, and (B) the acetylcholine receptor agonist carbachol on membrane potential (E_m) and intracellular pH (pH_i) of a leech neuropile glial cell. [From J. W. Deitmer, unpublished data.]

porter. With respect to the action of neurotransmitters on pH_i, glial cells appear to resemble neurons, while the glial Na^+-HCO_3^- cotransporter is reminiscent of epithelial cells.

The action of neuronal electrical activity, neurotransmitters and the H^+/HCO_3^--transporting carriers in the cell membranes produce pH shifts in neurons, extracellular space, and glial cells. It seems often necessary to monitor pH in *all three compartments* in order to understand the mechanisms of the acid and alkaline transients. We have begun to learn some of the mechanisms of these diverse and multiphasic pH changes. It is clear that glial cells regulate their pH_i, and make a significant contribution to the pH_o shifts, which feed back on neuronal and glial cell functions. For nearly all events related to pH changes in nervous tissue, the presence of HCO_3^- has a great impact; HCO_3^- determines the buffering power to a large extent, it enables HCO_3^- flux through $GABA_A$ receptor channels and thereby modifies the inhibitory synaptic potentials, and HCO_3^- is the substrate for the powerful glial electrogenic Na^+-HCO_3^- cotransporter. The maintenance of pH in neurons, glial cells and extracellular space, and the modulation of pH_o, plays an important and diverse role in the functioning of nervous systems; glial cells may take the part of a H^+ caretaker, similar as has been postulated for K^+, and some neurotransmitters and metabolites.

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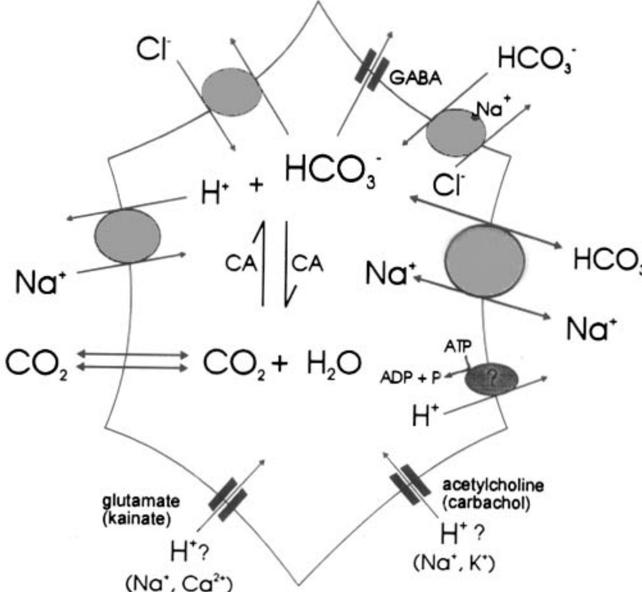

FIG. 14-6. Schematic summary of membrane channels and carriers relevant for pH_i in a hypothetical glial cell. The carbonic anhydrase (*CA*) catalyzes a fast equilibrium between $H^+ + HCO_3^-$ and $CO_2 + H_2O$, to which the cell membrane is permeable. An ATP-driven proton pump has been suggested, and the H^+ permeability of glutamate and acetylcholine receptor channels still needs to be shown more directly (hence marked with *question mark*). The Na^+-dependent $Cl^- - HCO_3^-$ exchange may actually transport Na^+. The steady-state pH_i, at least in some glial cell types, appears to be dominated by the electrogenic $Na^+ - HCO_3^-$ cotransport, which may operate as a voltage-dependent pH_i clamp.

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15 Transport of neuroactive amino acids in astrocytes

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All animal cells express a number of relatively specific transport systems for amino acids that are classified on the basis of Na^+ dependency and amino acid specificity (cf. Christensen, 1990). These transport systems are also found in brain cells (Sershen and Lajtha, 1979). Specific high-affinity transport systems for the neuroactive amino acids glutamate, aspartate, GABA, taurine, and glycine are present in the brain in addition to the above-mentioned amino transport systems (Sershen and Lajtha, 1979). The availability of preparations of neurons and astrocytes in primary cultures during recent years has made it possible to investigate in detail the properties of these amino acid transport systems at the cellular level (cf. Schousboe, 1982b). This chapter focuses on the properties of the astrocytic transport systems for the above-mentioned amino acids, but, for the sake of comparison, neuronal amino acid transport systems are also described.

EXCITATORY AMINO ACIDS

Glutamate and Aspartate

Kinetics and Substrate Specificity. The kinetic properties of glutamate uptake have been studied in a large variety of preparations of glial cells including bulk-prepared cells, cell lines, and primary cultures (cf. Schousboe, 1981, 1982b). Table 15-1 summarizes K_m and V_{max} values for cultured astrocytes and neurons. It can be seen that the glial uptake system exhibits high affinity for glutamate; if V_{max} values are compared with those determined in different neuronal preparations, including glutamatergic neurons, the conclusion is that glial cells and, in particular, astrocytes are more efficient than neurons with regard to glutamate transport (Hertz, 1979; Schousboe, 1981, 1982b; Hösli et al., 1986; Schousboe et al., 1988; Erecinska and Silver, 1990).

Detailed mutual inhibition and uptake studies of acidic amino acids with either carboxylic or sulfonic/sulfinic acidic groups have shown that in astrocytes L-glutamate, L-aspartate, cysteic sulfinate (CSA), and cysteate (CA) are likely to share the same carrier (Table 15-2). Apart from the finding that D-aspartate is a mixed competitive and noncompetitive inhibitor of L-glutamate uptake (Table 15-2), this amino acid also seems to share this carrier. In this context, it is interesting that the higher homologues of CSA and CA, homocysteic sulfinate (HCSA) and homocysteate (HCA) are not transported by the same carrier. This is, however, in line with the prediction (Schousboe et al., 1988) that in order for an acidic amino acid to bind efficiently to the carrier the two acidic groups must be close to each other. Molecular modeling of these molecules clearly illustrates this notion (Griffiths et al., 1989). Similar uptake and inhibition studies have been performed in cultured neurons and synaptosomes (Drejer et al., 1983b; Griffiths et al., 1989; Grieve et al., 1991; Rauen et al., 1992) and essentially the same pattern was observed. It should be noted, however, that in neurons and synaptosomes, D-aspartate is a competitive inhibitor of L-glutamate uptake (Drejer et al., 1982; Rauen et al., 1992), whereas as seen in Table 15-2 and confirmed in a recent study by Rauen et al., (1992), D-aspartate is either a noncompetitive or a mixed competitive/noncompetitive inhibitor in astrocytes. That the neuronal and astrocytic glutamate carriers may be kinetically different is also suggested by the following observations. β-Methylene-aspartic acid (β-MA) inhibits astrocytic D-aspartate or L-glutamate uptake noncompetitively (Bender et al., 1989; Dunlop et al., 1992) but inhibits neuronal D-aspartate uptake competitively (Dunlop et al., 1992). Moreover, L-*trans*-pyrrolidine-2,4-dicarboxylate (L-*trans*-PDC) has been shown to be a more potent inhibitor of synaptosomal glutamate uptake than of glial glutamate uptake when its efficacy was compared at a fixed glutamate concentration (Rauen et al., 1992). Surprisingly, however, when a kinetic analysis was performed, the K_i value for L-*trans*-PDC was higher in synaptosomes than in astrocytes, indicating that the compound is a more efficient inhibitor of astrocytic glutamate uptake (Rauen et al., 1992). In any event,

TABLE 15-1. *Kinetic Parameters for Glutamate or Aspartate Uptake into Different Preparations of Glial Cells and Neurons*

Cell Preparation	K_m (μM)	V_{max} (nmol × min^{-1} × mg^{-1})	Reference
Astrocytes			
C-6 astrocytoma	66	—	Henn et al. (1974)
	15	3.8	Faivre-Bauman et al. (1974)
MGM-LM glioma	20	2.7	Stewart et al. (1976)
NN glioma	14	0.7	Balcar et al. (1977)
	12–19	0.2–0.3	Balcar et al. (1978)
Neocortical prim. cult.	220	8.0	Schousboe et al. (1977b)
	30–90	30–75	Hertz et al. (1978a)
Prefront. cortical, prim. cult.	58	14	L. Drejer et al. (1982)
Occip. cortical, prim. cult.	33	11	Drejer et al. (1982)
Neostriatal prim. cult.	82	27	Drejer et al. (1982)
Cerebellar, prim. cult.	34	6	Drejer et al. (1982)
C-6 astrocytoma	22	12	Balcar et al. (1987)
Striatal prim. cult.	110	37	Hansson (1986)
Neocortical prim. cult.	84	14	Griffiths et al. (1989)
Neocortical prim. cult.	18	4	Flott and Seifert (1991)
Retinal, prim. cult.	134	21.5	Somohano and López-Colomé (1991)
Neurons			
Cerebellar cell line	50	0.6	Stallcup et al. (1979)
Neocortical, prim. cult.	43	5.9	Drejer et al. (1982)
Cereb. granule cells, prim. cult.	42	10.2	Drejer et al. (1982)
	36	9	Yu and Hertz (1982)
C-1300 neuroblastoma	20	0.4	Balcar et al. (1987)
Cereb. granule cells, prim. cult.	21	12	Griffiths et al. (1989)
Retinal, prim. cult.	13	2.4	Somohano and López-Colomé (1991)

Prim. cult., primitive culture; prefront., prefrontal; occip., occipital; cereb., cerebral.

there is reason to believe that the neuronal and astrocytic glutamate carriers may differ from each other, a notion borne out by recent cloning and immunohistochemical studies (Danbolt et al., 1992; Pines et al., 1992; Storck et al., 1992) as discussed below.

Ionic Requirements. Many studies have shown that glutamate uptake into astrocytes is sodium-dependent (cf. Schousboe, 1981, 1982b; Schousboe et al., 1977b, 1988; Erecińska and Silver, 1990) and the K_m for Na^+ is low (Schousboe et al., 1977b, 1988). There have been conflicting reports of the coupling ratio be-

TABLE 15-2. *Uptake and Mutual Inhibition of Acidic Amino Acids in Astrocytes*

Transported Amino Acid	Inhibiting Amino Acid						
	L-Glu	L-Asp	D-Asp	L-CA	L-CSA	L-HCA	L-HCSA
L-Glu	67[a]	68	106[b]	—	—	—	—
L-Asp	78	77[a]	70	—	—	—	—
D-Asp	75	82	83[a]	47	120	1560	2730
L-CA	—	—	—	88[a]	—	—	—
L-CSA	—	—	—		101[a]	—	—
L-HCA	—	—	—	—	—	901[a]	—
L-HCSA	—	—	—	—	—	—	225[a]

Astrocytes were cultured as described by Hertz et al. (1982, 1989b) and the kinetic analysis of uptake and inhibition performed as detailed by Drejer et al. (1983b), Griffiths et al. (1989), and Grieve et al. (1991), which also contain the original data.

Glu, glutamate; Asp, aspartate; CA, cysteic acid; CSA, cysteine sulfinic acid; HCA, homocysteic acid; HCSA, homocysteine sulfinic acid.

[a] K_m values (μM) for uptake.

[b] Mixed competitive/noncompetitive inhibition. Otherwise, the values indicate K_i values (μM) for competitive inhibition.

tween Na^+ and glutamate (cf. Erecińska and Silver, 1990), but this value is probably greater than unity, as indicated by studies of influx of $^{22}Na^+$ and glutamate simultaneously (Stallcup et al., 1979; Kimelberg et al., 1989). In addition to the Na^+-dependent high-affinity glutamate-uptake system, a Cl^--dependent component may be present in astrocytes, but it seems only to account for a minor fraction of the total glutamate uptake (Pin et al., 1984; Waniewski and Martin, 1986; Bridges et al., 1987; Flott and Seifert, 1991). These findings regarding sodium and chloride dependency of the astrocytic glutamate carrier are largely in line with what has been reported for neuronal glutamate uptake (cf. Schousboe et al., 1988; Erecińska and Silver, 1990). The glial glutamate carrier has also been shown to depend on K^+ and pH or bicarbonate (Sarantis and Attwell, 1990; Barbour et al., 1991; Bouvier et al., 1992). According to the model of Bouvier et al. (1992), glutamate is cotransported with two sodium ions, while one K^+ and one OH^- (or HCO_3^-) are countertransported, making the carrier highly electrogenic. Depolarization accordingly will reverse the carrier, leading to a large efflux of glutamate (Sarantis and Attwell, 1990; Szatkowski et al., 1990).

Molecular Characterization and Cloning. Recent cloning studies have established the existence of at least three glutamate carriers with different degrees of homology in the amino acid sequences (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992). The carrier cloned by Pines et al. (1992) has been shown by immunohistochemistry to be exclusively located in astrocytes (Danbolt et al., 1992). The carrier cloned by Kanai and Hediger (1992) from rabbit small intestine was shown by *in situ* hybridization studies to be also present in the brain (Kanai and Hediger, 1992). Its cellular localization was, however, not firmly established, but it appears to be present in neurons. When expressed in *Xenopus* oocytes it was shown to have the same basic properties with regard to substrate specificity as described above (Kanai and Hediger, 1992). Also the transporter cloned from the brain exhibits the kinetic pattern of the astrocytic glutamate carrier when incorporated into liposomes subsequent to extraction from HeLa cells transfected with the cDNA (Pines et al., 1992). Based on hydropathy analysis these two carriers (Pines et al., 1992; Kanai and Hediger, 1992) have been proposed to have different numbers of membrane-spanning domains, reflecting different amino acid sequences. Thus the glial glutamate carrier has been proposed to consist of eight putative membrane-spanning domains (Pines et al., 1992), whereas the carrier cloned from the rabbit intestine supposedly has ten such domains (Kanai and Hediger, 1992). Further cloning studies are likely to reveal additional subtypes of glutamate transporters in analogy to what has been demonstrated for glutamate receptors (cf. Gasic and Heinemann, 1991), although the heterogeneity of the carrier family may not turn out to be as complex as the heterogeneity of the receptor family.

Factors Regulating Carrier Expression. It is well documented by a number of studies (Schousboe and Divac, 1979; Drejer et al., 1982; Hansson, 1983, 1986) that astrocytic glutamate uptake differs with regard to capacity among astrocytes from different brain regions. It was originally suggested that such differences in the efficacy of glutamate uptake could be related to the role of astrocytes as the primary site for inactivation of glutamatergic activity as the uptake of glutamate was particularly pronounced in astrocytes originating from brain areas with a pronounced glutamatergic activity (Schousboe and Divac, 1979). It has subsequently been demonstrated that neuronally released macromolecular substances, presumably proteins, have the ability to increase the functional activity of glutamate carriers in astrocytes (Drejer et al., 1983a; Schousboe et al., 1986b). Future studies should be aimed at a better understanding of such processes as they could represent an important regulatory mechanism for astrocytic amino acid uptake (see Chapter 48, this volume).

INHIBITORY AMINO ACIDS

Gamma-Aminobutyric Acid

Kinetics and Substrate Specificity. Table 15-3 summarizes K_m and V_{max} values for GABA uptake into a variety of preparations of glial cells. K_m values vary in the range 1 to 50 μM, but clearly glial cells possess a high-affinity transport system for GABA. Comparison of GABA uptake into glial cells and neurons (cf. Schousboe 1981, 1982b; Hösli et al., 1986) shows that neurons have a higher capacity for GABA uptake than astrocytes. According to Hertz and Schousboe (1987) about 20% of GABA released from GABAergic neurons will be taken up into adjacent astrocytes, whereas the remaining 80% may be taken up into the nerve endings and hence will be available for release as a neurotransmitter. The demonstration that GABA analogues (cf. Figure 15-1 and Table 15-4), which preferentially inhibit glial GABA uptake, act as anticonvulsants (Horton et al., 1979; Frey et al., 1979; Krogsgaard-Larsen et al., 1981; Meldrum et al., 1982; Croucher et al., 1983; Wood et al., 1983; Schousboe et al., 1986a; Gon-

TABLE 15-3. *Kinetic Parameters for GABA Uptake into Different Preparations of Glial Cells and Neurons*

Cell Type	K_m (μM)	V_{max} (nmol × min^{-1} × mg^{-1})	Reference
Astrocytes			
C-6 astrocytoma	32	0.023	Schrier and Thompson (1974)
	0.22	0.0014	Hutchison et al. (1974)
	50	—	Henn (1976)
Chick embryo, prim. cult.	2.6	0.07	Percy et al. (1981)
Neocortical prim. cult.	40	0.35	Schousboe et al. (1977a)
	45	0.40	Hertz et al. (1978b)
	8.9	0.02	Balcar et al. (1979)
	2.3	0.05	Borg et al. (1980)
	31	0.33	Larsson et al. (1981)
	30	0.27	Larsson et al. (1983)
	24	0.87	Larsson et al (1985)
	25	0.85	Larsson et al. (1986a)
Cerebellar prim. cult.	80	0.18	Nissen et al. (1992)
Neurons			
Neuroblastoma	0.15	0.002	Hutchison et al. (1974)
Cerebellar prim. cult.			
Neocortical prim. cult.	9.1	0.6	Balcar et al. (1979)
	8.6	15.1[b]	Borg et al. (1980)
	8.1	1.6	Larsson et al. (1981)
Spinal cord, prim. cult.	3.7	—	Farb et al. (1979)
Chick embryo, prim. cult.	4.3	0.18	Percy et al. (1981)
Neocortical prim. cult.	8	1.5	Larsson et al. (1981)
	14	1.2	Larsson et al. (1983)
	21	2.8	Larsson et al. (1985)
	25	2.2	Larsson et al. (1986a)

Prim. cult., primitive culture.
[a]Value recalculated from nmol × mg^{-1} DNA on the basis of the DNA content in rat brain reported by Zamenhof et al. (1972).
[b]Expressed per mg DNA (mouse brain cultures).

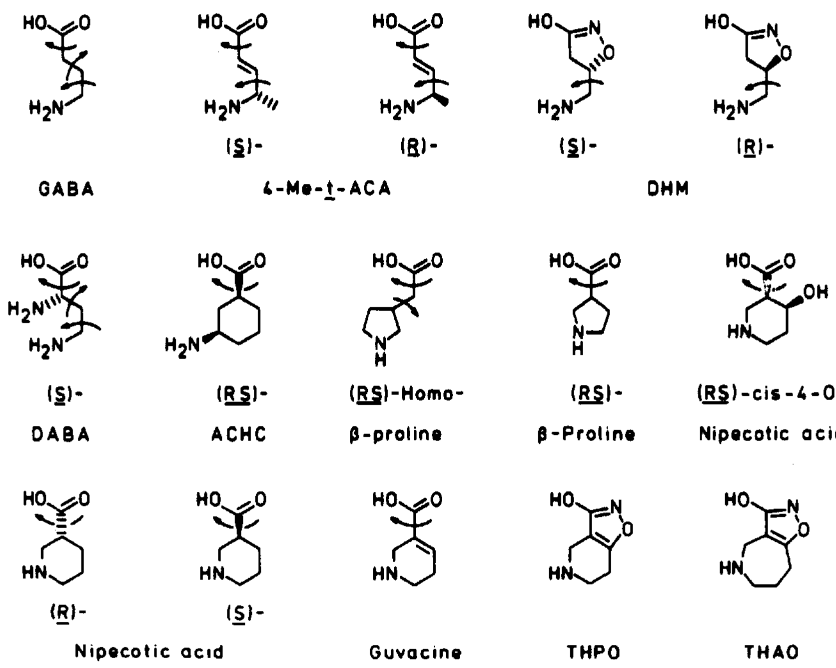

FIG. 15-1. Structural formulas with indications of structural conformational flexibility of GABA analogues, which inhibit high-affinity GABA uptake. [From Schousboe et al. (1991a), with permission.]

TABLE 15-4. *Kinetic Constants and Inhibition Patterns of GABA Analogues that Inhibit Glial and Neuronal GABA Uptake*

GABA Analogue	Inhibition					
	Glia			Neurons		
	K_i (μM)	Type	Substrate	K_i (μM)	Type	Substrate
GABA	30	C	+	25	C	+
(*RS*)-ACHC	700	N	+	70	C	+
(*RS*)-Homo-β-proline	16	C	n.d.	6	C	n.d.
(R)-Nipecotic acid	15	C	+	11	C	+
(*RS*)-*cis*-4-OH-nipecotic acid	147	N	+	55	C	+
Guvacine	28	C	n.d.	31	C	n.d.
THPO	550	C	—	a		
THAO	600	C	n.d.	a		
DPB-nipecotic acid	2	C	—	1	C	—
DPB-guvacine	4	C	n.d.	5	C	n.d.
DPB-THPO	26	C	n.d.	38	C	n.d.
DPB-THAO	3	C	n.d.	9	C	n.d.

Cells were cultured as described by Hertz et al. (1982, 1989a, 1989b) and the kinetic analyses of uptake and inhibition of GABA and its analogues were performed as described in the original publications (Schousboe et al., 1981, 1990a, 1991a; Larsson et al. 1981, 1983, 1985, 1988).

DPB, diphenylbutenyl derivatives of the parent compound; THPO, (4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol); THAO, (4,5,6,7-tetrahydroisoxazolo[4,5-*c*]azepin-3-ol); ACHC, *cis*-3-Aminocyclohexane carboxylic acid; C, competitive inhibition; N, noncompetitive inhibition; n.d., Not determined.

salves et al., 1989a, 1989b; White et al., 1993) indicates that uptake of GABA into astrocytes is of physiological and functional importance (cf. Schousboe et al., 1991a; Chapter 48, this volume). This has also been demonstrated in cocultures of GABAergic neurons and astrocytes in which administration of THPO (4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol) led to an enhanced depolarization coupled release of GABA (Westergaard et al., 1992).

Glial GABA uptake traditionally has been claimed to be selectively inhibited by β-alanine, and this amino acid has been widely used as a marker of glial GABA uptake (Iversen and Kelly, 1975; Schon and Kelly, 1975). Recent studies of the kinetics of uptake of GABA, β-alanine and taurine, as well as their mutual inhibition in synaptosomes, neurons, and astrocytes (Larsson et al., 1986b; Debler and Lajtha, 1987) have, however, questioned this dogma. As can be seen from Table 15-5, β-alanine in both cell types shares a transporter with taurine, which is distinct from that of GABA. This is apparent from the competitive nature of inhibition of taurine uptake by β-alanine and the noncompetitive nature of the corresponding inhibition of GABA uptake. On this basis, it may not be advisable to use β-alanine as the only experimental tool to identify and selectively investigate glial GABA transport sites.

Ionic Requirements. GABA transport in astrocytes is dependent on external sodium (Schousboe et al.,

TABLE 15-5. *Mutual Inhibition by GABA, Taurine, and β-Alanine of the Transport of the Same Amino Acids in Neurons and Astrocytes*

Amino Acid as Substrate	Amino Acid as Inhibitor					
	Neurons			Astrocytes		
	GABA	Taurine	β-Alanine	GABA	Taurine	β-Alanine
GABA	25[a]	15,500(N)	1,666(N)	31[a]	1,000[c]	843(N)
Taurine	911(N)[b]	20[a]	72(C)	>1,000[c]	25[a]	71(C)
β-Alanine	397(N)	217(C)	73[a]	1,222(N)	24(C)	71[a]

Mouse cerebral cortex neurons and astrocytes were cultured as detailed by Hertz et al. (1982, 1989a, 1989b) and uptake experiments performed as described by Larsson et al. (1986a), which also contain the original data.

[a] K_m (μM) values for uptake of the respective amino acid.

[b] The values indicated with (N) or (C) represent K_i (μM) values for the inhibition; N, noncompetitive inhibition by the amino acid acting as an inhibitor; C, competitive inhibition by the amino acid acting as an inhibitor.

[c] The type of inhibition is not clarified (A. Schousboe, unpublished observations).

1977a; Larsson et al., 1986a). It is interesting that the coupling ratio between Na^+ and GABA changes from unity to a value of 2 or more when astrocytes mature and differentiate in culture (Larsson et al., 1986a). Whether the same is true for astrocytes *in vivo* is not known, but if this is the case it has important functional implications for clearance of GABA from the extracellular environment. As the efficiency of the transport system greatly increases with an increase in the sodium coupling ratio (Martin, 1976; Schousboe, 1981), extracellular GABA levels are likely to be higher during early development of the central nervous system than at later more differentiated stages. Since GABA acts as a neuronal differentiation signal during early development (Redburn and Schousboe, 1987; Meier et al., 1991) such a relative inefficiency of cellular GABA uptake may be quite important during a period where overall GABA concentrations are low but where the extracellular availability of GABA may be of importance for normal neuronal development.

In addition to its dependence on sodium, GABA uptake is also chloride-dependent (Kanner, 1978) which contrasts with glutamate uptake (cf. above discussion). On the other hand, glial GABA uptake is not dependent on the presence of potassium (Hertz et al., 1978b) but is inhibited by depolarizing concentrations of potassium (Schousboe et al., 1977a). This is due to the fact that when GABA is cotransported with sodium the efficiency of the transport system decreases greatly with an increase in the membrane potential, that is, a depolarization (Martin, 1976; Schousboe, 1981).

Molecular Characterization and Cloning. A GABA transporter (GAT-1) was first cloned and sequenced by Kanner and coworkers (Guastella et al., 1990) and it was established that it belongs to a superfamily of neurotransmitter transporters, including those for dopamine and serotonin. They are characterized by 12 putative membrane-spanning domains (Guastella et al., 1990), a characteristic also proposed for the intestinal glutamate transporter but not for the glial glutamate transporter (cf. above discussion). Since the first cloning of a GABA transporter by Guastella et al. (1990), two other GABA-transporter clones (GAT-2 and GAT-3) have been reported (Borden et al., 1992). The cellular location of these transporters has so far not been firmly established, but the kinetic diversity of GABA-transporters described above clearly is supported by the results of the cloning experiments. These experiments do not, however, clarify the pharmacological distinction between neuronal and glial GABA uptake (Table 15-4), and it is not clear whether the GAT-1, GAT-2, and GAT-3 clones represent neuronal or glial GABA carriers (Borden et al., 1992).

Factors Regulating Carrier Expression. In analogy with what was reported for glial glutamate uptake (cf. above discussion), glial GABA uptake can be enhanced by neuronally released substances (Drejer et al., 1983a). Recently, a 30 kD glycoprotein has been purified from conditioned media from cultured cerebellar granule cells, which induces GABA carriers in astrocytes (Nissen et al., 1992) and hence is named GABA carrier-inducing protein (GABA-CIP). The inducing effect is indicated by the observation that the increase in V_{max} for GABA uptake seen after exposure of the cells to GABA-CIP could be prevented by the protein synthesis blocker actinomycin D (Nissen et al., 1992). This may represent an as yet unknown mechanism by which neurons may be able to regulate astrocytic functions pertinent to maintenance of neuronal signaling pathways.

Glycine

Kinetics and Ionic Requirements. High-affinity glycine uptake has been demonstrated in a number of neural preparations (cf. Schousboe, 1982b; Hösli et al., 1986). Detailed kinetic studies concerning astrocytic glycine uptake are, however, scarce but such an uptake system has been convincingly demonstrated (Wilkin et al., 1981; Hannuniemi and Oja, 1981; Zafra and Giménez, 1986; Reynolds and Herschkowitz, 1986; Hösli et al., 1986; Ottersen et al., 1987; Holopainen and Kontro, 1989). The kinetic characteristic of astroglial glycine uptake is similar to that found in neurons (Holopainen and Kontro, 1989). Inhibition studies have shown that glycine uptake is mediated by a specific carrier, which is different from those transporting other inhibitory neurotransmitter amino acids (Holopainen and Kontro, 1989). The glial glycine uptake is strictly Na^+-dependent and it is also Cl^--dependent, although this dependency is less pronounced than that for Na^+ (Holopainen and Kontro, 1989).

Taurine

Kinetics and Substrate Specificity. Since the first reports on astrocytic high-affinity taurine uptake (Borg et al., 1976; Schousboe et al., 1976), taurine uptake has been studied in a number of glial cell preparations and kinetic constants for glial taurine uptake have been summarized in Table 15-6. Also neurons of different phenotypes express a high-affinity carrier for taurine (Holopainen et al., 1983, 1984, 1987; Larsson et al., 1986b; Abraham and Schousboe, 1989;

TABLE 15-6. *Kinetic Parameters for Taurine Uptake into Different Preparations of Glial Cells and Neurons*

Cell Type	Kinetic Constants K_m (μM)	V_{max} (nmol × min^{-1} × mg^{-1})	Reference
Astrocytes			
C-6 astrocytoma	15	—	Henn (1976)
	10	0.39	Schrier and Thompson (1974)
	30	0.16	Sieghart and Karobath (1976)
NN-glial cell line	12	0.05	Borg et al. (1976)
Spinal glioma cell			
LRM 55	30	1.7	Martin and Shain (1979)
Neocortical prim. cult.	95	1.0	Schouseboe et al (1976)
	4.5	79.7[a]	Borg et al. (1980)
	38	0.2	Sanchez-Olea et al. (1991)
	39	0.3	Sanchez-Olea et al. (1992)
	18	1.0	Holopainen (1984)
Neurons			
Neuroblastoma	2.2	0.13	Richelson and Thompson (1973)
	18.7	0.23	Bort et al. (1976)
Neocortical prim. cult.	6.0	5.8 [a]	Borg et al. (1980)
Cerebellar granule neurons	15	0.2	Abraham and Schousboe (1989)
	111	0.6	Schousboe et al. (1991b)

Prim. cult., primitive culture.
[a]Per mg DNA

Schousboe et al., 1991b). Taurine uptake is inhibited by structural analogues of taurine (Holopainen, 1984; Holopainen et al., 1983, 1984, 1987) most notably by β-alanine (Table 15-5) and guanidino ethane sulfonic acid (GES). The actions of the latter compound have recently been extensively reviewed (Huxtable, 1992). It is important to stress that taurine and GABA, which share certain inhibitory properties (cf. Hösli and Hösli, 1978; Huxtable, 1989), do not share the same carrier, as evidenced by the kinetic studies summarized in Table 15-5.

Ionic Requirements. Studies of taurine uptake in astrocytes have shown that the carrier is dependent on both Na^+ and Cl^- for its action (Schousboe et al., 1976; Holopainen et al., 1987; Holopainen, 1988). The sodium dependency is also confirmed by the observation that taurine uptake into C-6 glioma cells is inhibited by ouabain (Sieghardt and Karobath, 1976). The coupling ratio between sodium and taurine has been reported to be close to unity (Schousboe, 1982a), but recent experiments (A. Schousboe, unpublished observations) have indicated that this coupling ratio is closer to 2 in more differentiated astrocytes. In analogy to what has been found for GABA uptake, taurine uptake is inhibited by high concentrations of K^+ due to the depolarization of the cells (Schousboe et al., 1976).

Molecular Characterization and Cloning. Recent cloning studies (Liu et al., 1992; Smith et al., 1992) have shown that the taurine carrier from rat or mouse brain is structurally related to the GABA carrier, that is, the protein has 12 putative membrane-spanning segments. It should be noted, however, that the cDNAs for the GABA and taurine carriers only exhibit partial homology with about 60% nucleotide identity. The reconstituted carrier was inhibited by β-alanine, confirming kinetic studies on taurine transport in intact brain cells (cf. above discussion). These cloning studies have also underlined the conclusion from previous studies of the kinetic properties of GABA and taurine uptake (cf. above discussion) that these two amino acids are transported by distinctly different carriers.

Taurine Fluxes and Cell Swelling. It has recently been convincingly shown that astrocytic (and neuronal as well) cell swelling and the subsequent regulatory volume decrease (cf. Kimelberg and Ransom, 1986) is associated with a pronounced efflux of taurine and to a lesser extent of some other amino acids (Pasantes-Morales and Schousboe, 1988, 1989; Kimelberg et al., 1990; Martin et al., 1990; Pasantes-Morales et al., 1990; Schousboe et al., 1990b; Schousboe and Pasantes-Morales, 1992). Although this efflux of taurine does not appear to be mediated directly by

the taurine carrier (Sanchez-Olea et al., 1991, 1992; Schousboe et al., 1991b; Pasantes-Morales et al., 1993), the taurine carrier is of functional importance for this process to occur. The basis for this role of taurine in cell volume regulation is its high intracellular concentration, which is secured by the activity of the carrier (cf. Sanchez-Olea et al., 1991).

NEUROTRANSMITTER-RELATED AMINO ACIDS

Glutamine

Glial glutamine transport has been investigated in different preparations, including bulk-prepared cells and cultured astrocytes (Weiler et al., 1979; Schousboe et al., 1979; Ramaharobandro et al., 1982). Values for K_m and V_{max} vary considerably between these studies, but uptake rates calculated at an extracellular glutamine concentration of 100 μM are quite similar, being about 1 nmol × min^{-1} × mg^{-1} protein (Schousboe et al., 1988). By comparison, both GABAergic and glutamatergic neurons appear to have similar capacities for glutamine uptake as those reported for astrocytes (Schousboe et al., 1988). The substrate specificity of the glutamine carrier suggests that it may serve as a transporter for several amino acids such as alanine, leucine, and arginine (Christensen, 1990; Erecińska and Silver, 1990). There are only few studies available concerning the ionic requirements for glutamine uptake. Neurons and synaptosomes exhibit Na^+ dependency (Ramaharobandro et al., 1982; Erecińska et al., 1990), but no consistent data showing sodium dependency seem available for glial glutamine uptake.

Arginine

Remarkably little is known about how L-arginine is transported into brain cells. Synaptosomes have been shown to transport arginine and to subsequently metabolize it into ornithine, which can function as a precursor for glutamate, GABA, and proline (Johnson and Roberts, 1984). Arginine has recently attracted considerable attention as a precursor for the highly diffusible molecule nitric oxide, which is likely to be involved in signal transduction in the central nervous system (Garthwaite, 1991; Snyder and Bredt, 1991). In the light of a possible important role of arginine transport for the regulation of synthesis of nitric oxide, studies have been performed to characterize the transport of arginine into cultured astrocytes (Wiesinger, 1992) and into cultured neurons (Westergaard et al., 1993a). The K_m values are around 50 to 100 μM and the uptake of arginine in the two cell types is largely sodium-independent but strongly inhibited by depolarizing concentrations of potassium. The pattern of inhibition by basic L-amino acids is consistent with a "y^+" transport system (Christensen, 1990). In the light of the importance of arginine as a precursor for nitric oxide, it is of interest that the two nitric oxide synthase inhibitors N^G-monomethyl-L-arginine and N^G-amino-L-arginine have been shown to be potent inhibitors of arginine uptake into cultured neurons (Westergaard et al., 1993a).

Alanine

Alanine uptake has been demonstrated in brain slices (Cohen and Lajtha, 1972; Sershen and Lajtha, 1979) and in nerve terminal-enriched material (Shank and Campbell, 1982) but very little is known about alanine uptake into cultured astrocytes and neurons. Recently, Westergaard et al. (1993b) have reported kinetic constants for alanine uptake in astrocytes (K_m ~ 450 μM; V_{max} 8 nmol × min^{-1} × mg^{-1}) and neurons (K_m ~ 275 μM; V_{max} 16 nmol × min^{-1} × mg^{-1}), indicating that alanine uptake is more efficient into neurons than into astrocytes. The uptake was dependent on sodium with a K_m value for sodium around 5 mM in both cell types. The observed inhibition by high concentrations of glycine, valine, and glutamine is compatible with the notion that the alanine carrier present in these cultured cells represents the sodium-dependent carrier for small neural amino acids (Christensen, 1990). In this context it should be noted that the preferential uptake of alanine into neurons supports the notion that alanine may play an important role as an amino group donor for biosynthesis of neurotransmitter glutamate from α-ketoglutarate (Peng et al., 1991; Hertz et al., 1992; Schousboe et al., 1992a, 1992b; Schousboe and Westergaard, 1993).

CONCLUDING REMARKS

It is clear that astrocytes express high-affinity carriers for the amino acids that are either neurotransmitters or neuromodulators in their own right or serve as precursors for these substances. Since astrocytes are in close anatomical contact with synapses, it is very likely that these cells serve important roles with regard to supply of transmitter precursors to neurons and with regard to inactivation of the neurotransmitter amino acids. This latter function appears to be of particular physiological importance in case of the excitatory amino acids glutamate and aspartate. Maintenance of a low extracellular concen-

tration of these amino acids (below 5 μM) appears to be of utmost importance for protection of neurons against cytotoxic actions of these amino acids (Nicholls and Attwell, 1990) and the highly efficient high-affinity glutamate-aspartate carrier in astrocytes is well suited to serve this purpose. Although astrocytic glutamate uptake is superior to that present in nerve cells, it should be emphasized that the neuronal glutamate uptake is in no way to be regarded as functionally unimportant. In glutamatergic neurons it may ensure that released glutamate can be taken up into the nerve ending and reutilized as a neurotransmitter. In this context it should be emphasized that, although glutamate can to some extent be reutilized as a neurotransmitter, a considerable drain from the neurotransmitter pool occurs. Therefore uptake of other precursors such as glutamine, alanine, and α-ketoglutarate is important (Hertz et al., 1992; Schousboe et al., 1992b; Schousboe and Westergaard, 1993). Since these metabolites are all primarily synthesized in astrocytes, these cells are of fundamental importance for supply of precursors for synthesis of neurotransmitter glutamate in neurons. In other types of neurons the uptake of glutamate could well be a mechanism by which the concentration of glutamate in the microenvironment of cells is kept at a low level. The reutilization of amino acid neurotransmitters by neurons appears to be a very important principle in the case of GABA. In this case astroglial GABA uptake may prevent reuse of released GABA, and hence partial inhibition of glial GABA uptake may enhance the inhibitory activity of GABAergic neurons and thereby prevent seizures.

The observation that astrocytes as well as neurons have a high-affinity uptake system for arginine is interesting, as arginine is the precursor to nitric oxide, an important signaling molecule in the brain (Snyder and Bredt, 1991). The finding that some of the inhibitors of nitric oxide synthase are potent inhibitors of the arginine transporter may point to the possibility of developing drugs affecting nitric oxide synthesis by acting as inhibitors of arginine uptake, which appears to be rate-limiting for nitric oxide synthesis (Westergaard et al., 1993a).

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16 Na,K-ATPase and its isoforms

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(Na^+ + K^+)-stimulated adenosine triphosphatase (Na,K-ATPase, or sodium pump) is a protein embedded in the plasma membrane. It catalyzes the active uptake of K^+ and extrusion of Na^+ at the expense of hydrolyzing ATP to ADP and P_i. This uphill transport establishes steep concentration gradients for the ions. The gradients are then harnessed by other proteins for a variety of essential functions, including electrical potential changes mediated by ion channels, the uptake of molecules like neurotransmitters, and the extrusion of Ca^{2+}. The Na,K-ATPase is phylogenetically ancient but is not found in protists or plants. Where it is found, it is always inhibitable by a class of natural toxins, the cardiac glycosides, such as digitalis and ouabain.

ENZYMATIC CHARACTERISTICS, AND THEIR IMPLICATIONS FOR FUNCTION IN GLIA

Active Na^+ and K^+ transport carried out by the Na,K-ATPase has certain basic characteristics in all well-studied cell types (Jørgensen, 1986). All cells require Na,K-ATPase activity to maintain their ion gradients. The amount of Na,K-ATPase activity found varies markedly, however, and typically reflects the ion flux work load. Astrocytes, like neurons, muscle, and transporting epithelia, have a relative abundance of the enzyme; fibroblasts and many other cell types have much less.

Hydrolysis of ATP by the Na,K-ATPase is accompanied by sequential protein conformation changes that translocate the ions (Jørgensen, 1986). Figure 16-1 shows a schematic diagram. Binding of Na^+ at the intracellular face accelerates the transfer of the terminal phosphate of ATP to an aspartate side chain in the active site, and this brings about a large conformation change that delivers Na^+ to the outside of the cell. Binding of K^+ then causes a conformation change that accelerates the hydrolysis of the covalently bound phosphate, and the K^+ is transported to the inside of the cell as the enzyme returns to its initial conformation. The principal enzyme conformations are known as E1 and E2; E1 is found in the presence of Na^+ and E2 in K^+, but the phosphorylated form of E2 (E2-P) is found in the presence of Na^+, Mg^{2+}, and ATP. The gene family of ion-transporting ATPases is sometimes referred to as the P-type ATPases (for their covalent phosphorylation at the active site) or the E1-E2 ATPases (for their characteristic conformation changes).

Although no crystal structure is available for the sodium pump, much is known about the enzymatic mechanism (Jørgensen and Andersen, 1988). Both Na^+ and K^+ can be demonstrated to be "occluded" at some stage of the turnover cycle: that is, caught within the membrane protein with no access to either side. The protein does not rotate within the membrane; instead ions are believed to move between membrane-spanning protein segments. These transmembrane segments may shift relative to one another, and they may rise or sink somewhat with respect to the plane of the membrane.

The whole turnover cycle is very slow compared to conductance through ion channels. When going at full speed, the enzyme will complete one turnover in 6 to 10 msec, moving $3Na^+$ in exchange for $2K^+$. The fact that one net positive charge is moved out of the cell makes the pump electrogenic. The cell compensates for the slowness of each Na,K-ATPase unit by making a lot of them. In excitable cells it is one of the most abundant plasma membrane proteins. Collectively, the pumps in a cell can make a direct contribution of 1 to 5 mV (hyperpolarization) to the membrane potential.

Affinities for the substrates (Na^+, K^+, and Mg·ATP) have been measured with purified enzyme (Jørgensen, 1986). A salient feature is that the affinities for all of the ligands are affected by the concentrations of the others and by the conformational state, leading to many apparent discrepancies in the literature. Nonetheless, it is reasonable to state that typical concentrations for half-maximal activation of ATPase activity are 5 to 10 mM for Na^+; 1 mM for K^+, and 0.3 mM for Mg·ATP. This means that extracellular K^+ (3.6 mM or more) and intracellular ATP (2 to 3 mM) are much less likely to be rate-limiting for enzyme activity than intracellular Na^+ (5 to 10 mM). In fact, there is much experimental evidence [mostly in transporting epithelia, liver, and cardiac

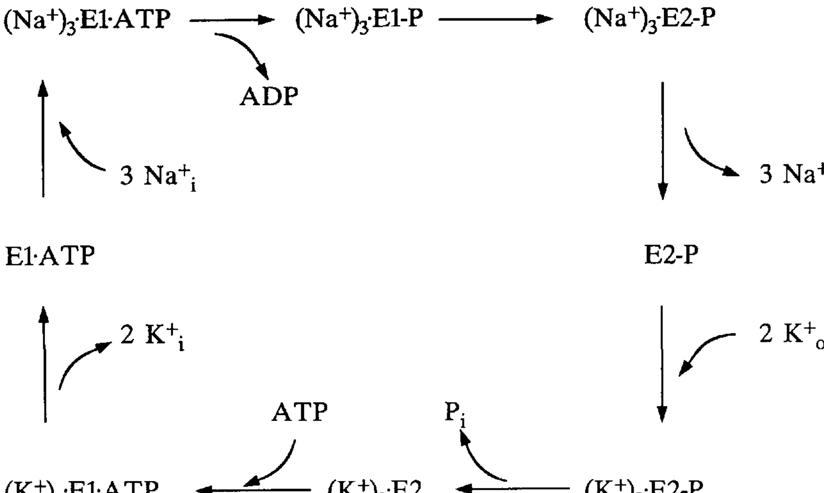


FIG. 16.1 Conformational changes of the Na,K-ATPase. This is a simplified scheme showing how the Na,K-ATPase couples the energy of ATP hydrolysis to the movement of ions across the membrane.

myocytes, but also in synaptosomes (Erecinska, 1989)] that elevation of intracellular Na^+ directly results in an increase in Na,K-ATPase activity. In measurements of pump current in intact cardiac cells, it has also been shown that affinities for extracellular K^+ (0.2 to 1.5 mM depending on conditions) are very similar to those measured with purified enzyme (Nakao and Gadsby, 1989).

Potassium ions are close to electrochemical equilibrium when the interior of the cell is negative, and so it does not cost the sodium pump very much thermodynamic energy to move K^+ from one side of the membrane to the other.* In contrast, Na^+ is far from its electrochemical equilibrium potential, since its diffusion into the cell is favored by both the concentration gradient and the voltage difference established by the open K^+ channels. This makes the active transport of Na^+ back out of the cell very energetically unfavorable. Na^+ transport should be less unfavorable during the transient depolarization of excitable cells. It has been demonstrated that the pump current (measured with whole-cell patch clamp recording) is actually voltage-dependent (De Weer et al., 1988). It is the translocation of Na^+, rather than K^+, that shows sensitivity to the membrane potential. The initial steps (the binding and occlusion of Na^+) are not very sensitive, but the release of Na^+ to the outside of the cell is (De Weer et al., 1988). In principle, if a hyperpolarized glial cell is depolarized by any means, one would predict that its Na,K-ATPase activity would increase even in the absence of changes in the saturation of its Na^+ and K^+ binding sites. The pump current in cardiac myocytes, for example, more than doubles between −85 mV and 0 mV (Figure 16-2) (Gadsby and Nakao, 1989).

Electrophysiological studies of pump activity have led to an explicit prediction about the structure of the enzyme. The Na,K-ATPase behaves quantitatively as if there were an "ion well," subject to membrane po-

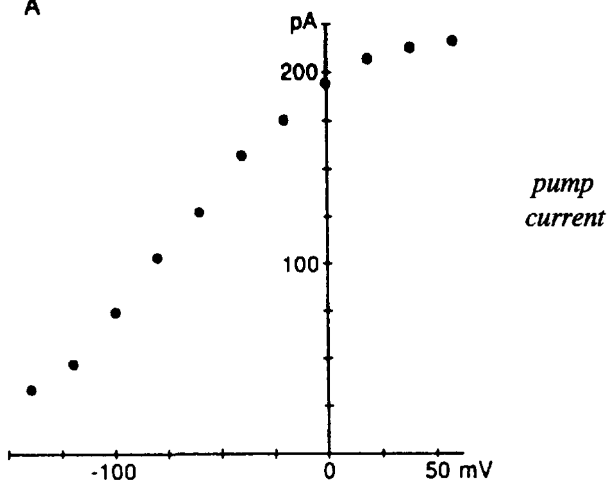


FIG. 16-2. Voltage-dependence of sodium pump current. Pump current was measured in cardiac myocytes using whole cell patch clamp. The current due to the Na,K-ATPase was assessed by subtracting values obtained with and without strophanthidin (a ouabain analogue). Conductance through various other pathways was minimized with TEA, Ba^{2+}, Cd^{2+}, and the removal of extracellular Ca^{2+}. Transmembrane voltage was changed in steps (millivolts; mV) and the resulting strophanthidin-sensitive current is plotted (picoamps; pA). For this experiment, intracellular (pipette) Na^+ was 50 mM, while extracellular Na^+ was 150 mM and extracellular K^+ was 5.4 mM. [From Gadsby and Nakao (1989), with permission.]

*The fundamental basis for electrical excitability is first, that gradients for Na^+ and K^+ are established across the membrane, and second, that a resting membrane potential is generated by opening K^+ channels, but not Na^+ channels. K^+ then diffuses down its concentration gradient (leaving its counterion behind) until the separation of charge produces enough of an electrical force to oppose further diffusion.

tential, within the protein but connected to the extracellular surface (Läuger, 1991a). Ions apparently move part of the way across the membrane between transmembrane segments in a narrow channel. This channel leads to a tighter region, deep within the protein, where ions are bound and conformation changes control ion translocation. Figure 16-3 illustrates this concept diagrammatically.

In summary, the properties of the prototypical sodium pump include (1) that intracellular Na^+, not extracellular K^+, is normally rate-limiting for turnover, and (2) that voltage dependence is a factor in cells with high and variable membrane potentials. Both of these enzymological features may affect how the Na,K-ATPase functions in glia.

DOES THE Na,K-ATPase MEDIATE POTASSIUM ION CLEARANCE?

As described in more detail in Chapter 31, astrocytes have an essential role in clearing the K^+ that is released from neurons into the extracellular cleft during activity. It has been proposed that the Na,K-ATPase directly takes up the K^+ (reviewed in Stahl, 1986; Sweadner, 1989; Reichenbach et al., 1992). This would be in contrast to K^+ uptake primarily by channels or carriers (refer to Chapters 11, 13, and 18), in which the Na,K-ATPase would play only a supporting role.

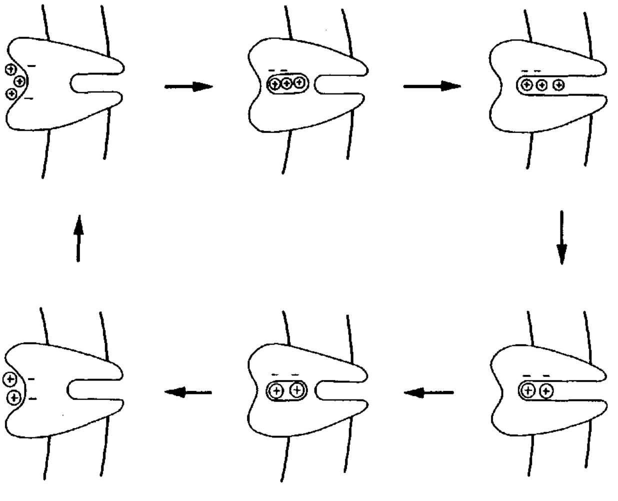


FIG. 16-3. An ion well within the Na,K-ATPase. Voltage-dependent properties of the Na,K-ATPase predict that ions must diffuse part way through the membrane in a pore in the protein, influenced by the membrane potential. The pore is presumably lined by transmembrane segments, and may be the site of cardiac glycoside binding. The intracellular surface is to the left and extracellular surface to the right. [From Läuger (1991b), with permission.]

There is a precedent for a role for the Na,K-ATPase in K^+ clearance elsewhere in the body (Moore, 1983). Serum K^+ levels would increase significantly after eating a meal if there were no mechanism for clearing the newly ingested K^+. The role of the Na,K-ATPase differs from its proposed role in glial-mediated K^+ clearance, however, because raised serum K^+ is not sufficient by itself to increase the activity of the pump. Instead, the signal is a hormone. Elevated serum K^+, like elevated glucose, stimulates the release of insulin from the β cells of the pancreas. Insulin then acts on skeletal muscle, liver, and fat to increase the activity of the Na,K-ATPase. The pump takes up the K^+ and stores it (predominantly in skeletal muscle) as a reservoir for later buffering of serum K^+ levels. Stimulation of ion transport is one of the most rapid of the many effects of insulin, but the mechanism is still debated (Guidotti, 1990). In liver and skeletal muscle, insulin increases intracellular Na^+ levels, thus activating the Na,K-ATPase by increasing the availability of the rate-limiting substrate inside the cell. In addition, insulin seems to increase Na,K-ATPase V_{max} by mechanisms that are not yet understood, possibly including recruitment of intracellular Na,K-ATPase pools to the plasma membrane.

Can the Na,K-ATPase mediate K^+ uptake in astrocytes directly in response to elevated extracellular levels? Its enzymatic properties must be considered. First, for an increase in extracellular K^+ to activate the Na,K-ATPase, the binding of K^+ at its extracellular sites would have to be on the steep part of the binding curve. This means that the enzyme should have a lower K^+ affinity than is usually observed in studies of purified enzyme. Second, the concentration of Na^+ inside the cell would have to be high enough to not be rate-limiting. For astrocyte Na,K-ATPase to be responsible for K^+ uptake then, it might be quite different from the enzyme in other tissues: higher Na^+ affinity, and/or lower K^+ affinity.

Intracellular Na^+ activity in resting mammalian astrocytes has been estimated to be 20 to 25 mM (Ballanyi et al., 1987), which is higher than in most cell types (see Chapter 18, this volume). Theoretically Na^+ may also enter locally through Na^+ channels, Na^+:transmitter uptake systems, or Na^+:K^+:Cl^- cotransport, supplying the Na,K-ATPase with enough Na^+ to support the exchange for extracellular K^+. Na^+ may also be supplied to some extent by cell coupling. Injection of current carried by Na^+ into mouse oligodendrocytes causes a passive exit of K^+, as measured with intracellular K^+ electrodes, and after the current pulse, K^+ levels were observed to be restored

via the ouabain-sensitive Na,K-ATPase (Kettenmann et al., 1987). Interestingly, recovery was similar if Li^+ or tetramethylammonium ion was ionophoresed into the cells instead of Na^+, which led to the suggestion that it was either the decrease in intracellular K^+ or the decrease in membrane potential, rather than the increase of intracellular Na^+, that activated the pump. With tetramethyammonium ion injection, however, recovery could be blocked by removing Na^+ from the extracellular fluid, implying that Na^+ had to enter to activate the Na,K-ATPase.

Evidence that astrocyte Na,K-ATPase has an unusually low affinity for K^+ has been reported (Franck et al., 1983; Guillaume et al., 1990; Reichenbach et al., 1992), although evidence for normal, high K^+ affinity has been published by other investigators (Kimelberg et al., 1978; Averet et al., 1987; reviewed in Stahl, 1986; Sweadner, 1989)]. Unpublished results from this laboratory, obtained with partially-purified Na,K-ATPase from cultured astrocytes, shows K^+ affinity as high as that of enzyme from axolemma. More physiological techniques, such as whole-cell patch-clamp, need to be applied to resolve the discrepancies. There are reports of cardiac glycoside-sensitive ATPase activity that is independent of Na^+ and/or K^+ in cultured astrocytes (Sweadner, 1983) and in retinal Müller cells (Reichenbach et al., 1987). Whether this is a related, but undiscovered, ATPase or a modification of the Na,K-ATPase remains to be determined, as well as its role in glial physiology.

A consequence of a large activation of the Na,K-ATPase by extracellular K^+ would be to hyperpolarize, not depolarize the cell, because the enzyme is electrogenic. *In vivo,* elevation of extracellular K^+ depolarizes glia, but a hyperpolarizing effect, which may be due to Na,K-ATPase, was uncovered when Ba^{2+} was used to block K^+ channels (Ballanyi et al., 1987).

The possibility that K^+ clearance is mediated by rectifying K^+ channels and/or a Na^+:K^+:Cl^- cotransport carrier is still quite strong. The Na,K-ATPase in glia would always be needed to extrude the Na^+ that comes in via other proteins such as Na^+-dependent neurotransmitter uptake systems and Na^+ channels (see Chapter 15). The open question is whether the Na,K-ATPase in astrocytes is specialized for a role in K^+ clearance, and quantitatively how important this is relative to the other mechanisms. As detailed below, astrocytes express the same isoforms of Na,K-ATPase α subunit and perhaps β subunit that are found in kidney, skeletal muscle, and many neurons.

ISOFORMS OF THE Na,K-ATPase

The Na,K-ATPase has two kinds of subunits, a 112,000 Dalton subunit called α, and a smaller glycoprotein subunit (32,000 Dalton of protein) called β (Figure 16-4). The α subunit has the binding sites for ATP and cardiac glycosides, and shows regions of sequence homology with a large family of ion-transporting ATPases, even those in protists (Lingrel et al., 1990). The β subunit is known to play a role in the biosynthesis and targeting of the enzyme to the plasma membrane, but its role in enzyme activity is not well understood. The two subunits have never been separated without irreversible loss of activity, however, and α synthesized in the absence of β is not active. Dimers of α and β are active, but there is experimental evidence to suggest that dimers may also associate to form functional tetramers.

There are three well-characterized isoforms of the α subunit, known as α1, α2, and α3 (Sweadner, 1989). Each is the product of a different gene, on different chromosomes (Lingrel et al., 1990). The genes show about 85% identity between isoforms, but each isoform shows 90 to 99% identity between vertebrate species (Takeyasu et al., 1990). All of the isoforms catalyze Na^+ and K^+-dependent ATPase activity, and all are inhibited by cardiac glycosides. The β subunit is also found in several isoforms derived from separate genes: two have been described in mammals and birds (β1 and β2) and a third in amphibians (β3). The sequences of the β subunit isoforms are more divergent than for the α isoforms: typically only 35 to 40% identity. There are also other closely related ATPases, such as H,K-ATPases, which have homologous α and β subunits, but these have not yet been described in glia.

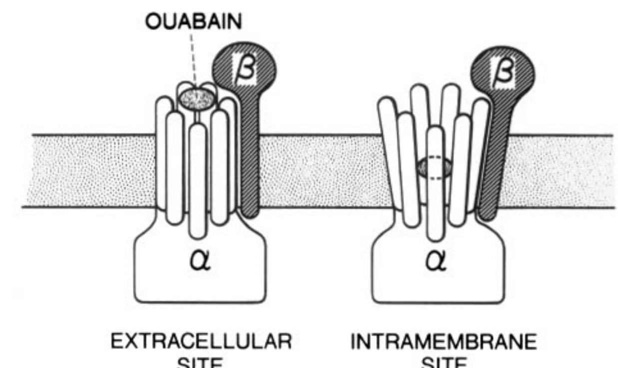


FIG. 16-4. Subunit composition of the Na,K-ATPase. The Na,K-ATPase α subunit spans the membrane multiple times, and has the binding sites for ATP and ouabain. The β subunit spans the membrane only once, and exposes most of its mass to the extracellular surface. Homologous ATPases like Ca^{2+}-ATPase do not have β subunits, but Na,K-ATPase will not fold correctly without one. The figure contrasts two possible locations for the location of the ouabain binding site: extracellular and between transmembrane segments. [From Sweadner (1993), with permission.]

There is abundant evidence, obtained with both isoform-specific antibodies and isoform-specific mRNA probes, that the expression of the Na,K-ATPase isoforms is tissue-specific and cell type-specific (Sweadner, 1989; Lingrel et al., 1990). Not surprisingly, the brain expresses all of the isoforms. More than one isoform is frequently expressed in the same cell simultaneously. If any α type can associate with any β type, then six different combinations are possible for mammals. It is quite likely that additional β subunits will be discovered.

CARDIAC GLYCOSIDE AFFINITY DIFFERENCES: A USEFUL TOOL

The α isoforms can differ in their ouabain affinity, depending on the animal species (Sweadner, 1989; Lingrel et al., 1990). In the rat, affinities for α1 are approximately 3×10^{-5}M; for α2, 10^{-7}M; and for α3, 3×10^{-8}M. Measurement of the binding of [^{3}H]ouabain to the lowest affinity form, α1, is for all practical purposes impossible because of its rapid dissociation during washes, and so detection of specific [^{3}H]ouabain binding in preparations derived from the rat indicates the presence of α2 or α3.

The molecular basis of the difference in ouabain affinity is now well understood (Lingrel et al., 1990; Sweadner, 1993). Substitution of one or a few critical amino acids in an extracellular segment of the α subunit, or the nearby transmembrane segments, is all that is needed to convert α1 with a low affinity (from a species like rat) to α1 with a high affinity (like those from species like sheep or human). Substitution of the β subunit is without effect.

The apparent ouabain affinities actually vary depending on the experimental conditions, since ouabain binding is highly dependent on the conformational state of the enzyme. Ouabain binding affinity is reduced by elevated extracellular K^+, for example, and binding will not occur to a significant extent in conditions where the enzyme cannot become phosphorylated at the active site (i.e., in the absence of ATP or P_i).

In preparations containing more than one isoform, low concentrations of ouabain (10^{-6} to 10^{-5} M) can be used to selectively inhibit α2 or α3. The relative contributions of α2/α3 and α1 are then estimated by subtraction. With intact cells, however, this experimental approach can be problematical, since inhibition of the high-affinity ATPase activity perturbs the intracellular Na^+ concentration and complicates the interpretation (McGill, 1991; McGill and Guidotti, 1991).

Na,K-ATPase ISOFORM EXPRESSION IN GLIA

The existence of Na,K-ATPase isoforms might explain some of the puzzling controversies in the literature if they have different functional properties. All of the five known Na,K-ATPase subunit types are found in the nervous system, and four out of five have been detected in glia: α1, α2, β1, and β2. Na,K-ATPase isoform expression in the nervous system has been reviewed (Sweadner, 1989, 1991, 1992; B. Schneider, 1992).

α Isoforms in Glial Cell Cultures

As shown below, astrocytes *in situ* can be demonstrated to express both α1 and α2 isoforms of the Na,K-ATPase. Glial cultures can be prepared that express only α1, or both α1 and α2. Early studies were done with cortical glial cultures from newborn rodents. Two criteria were used for detecting isoforms: the presence of low-affinity (α1) and high affinity (α2 and/or α3) ouabain sites, and the presence of two closely spaced bands of α subunit on SDS gels.* Marks and Seeds (1978) observed that in reaggregating mouse brain cell cultures, where both neurons and glia survived, both low and high-affinity sites were present when measured by the inhibition of Na,K-ATPase activity and by the inhibition of K^+ uptake. Conversely, in monolayer cultures, where neurons did not survive, only the low-affinity site was detected by ATPase assay or high affinity [^{3}H]ouabain binding. Sweadner (1979) found only low-affinity ouabain sites in rat cortical glial cultures as measured by inhibition of ATPase activity, and in addition, only the α1 electrophoretic band was detected on SDS gels. Membranes from whole brain had two ouabain affinities and two electrophoretic bands. Inoue et al. (1988), like Marks and Seeds (1978) detected only low-affinity ouabain sites in rat astrocyte cultures, both by inhibition of ATPase activity in membranes and of K^+ influx in cells, while neuronal cultures had both sites. Markwell et al. (1991) confirmed the virtual absence of high-affinity

*It is commonly observed that α1 migrates faster than α2 and α3 on SDS gels (Sweadner, 1979), although its true molecular weight is actually slightly higher. This property has sometimes been used successfully as a tool for isoform detection. It is also true, however, that apparently homogeneous α subunit types can be induced to split into two bands in different experimental conditions (Sweadner, 1990). The anomalous migration is thought to be due to peculiarities of SDS binding to hydrophobic stretches in the protein. Gel doublets of α are not sufficient proof by themselves of the presence of two isoforms without other, independent evidence.

[^{3}H]ouabain sites for glial cultures derived from either cortex or hippocampus of the mouse. They found that glia alone had only 4 to 8% of the specific [^{3}H]ouabain binding found in cultures that were additionally seeded thinly with neurons. Treatment with NMDA to kill neurons resulted in loss of about half of the [^{3}H]ouabain binding after 5 days. All these studies suggested that only α1 is found in cultured glia.

Other investigators, however, reported finding both high- and low-affinity ouabain sites in cultured glia. Walz and Hertz (1982) observed ouabain inhibition of K^+ uptake into mouse cortical astrocyte cultures at both low and high concentrations. Astrocytes grown in the presence or absence of dibutyryl cAMP for 2 to 3 weeks (to promote morphological differentiation) had similar biphasic inhibition curves (Walz and Hertz, 1982). Atterwill et al. (1984) also found both high- and low-affinity sites in astrocyte cultures, as well as in freshly isolated enriched astrocyte preparations. Both groups' observations had one thing in common: the high-affinity component was detected when cells were either loaded with Na^+ prior to assay, or preincubated with ouabain for 30 minutes, which would also elevate intracellular Na^+. This would maximize the activity of the Na,K-ATPase and also increase the rate at which low concentrations of ouabain saturate the high-affinity sites. Thus these studies raise the possibility that cultured glia may express both α1 and α2, but that α2 is only detected in some assays. This does not explain why no α2 was seen on gels (Sweadner, 1979) or why no high-affinity site was seen in ATPase assays of membrane preparations when Na^+ was not rate-limiting (Marks and Seeds, 1978; Inoue et al., 1988).

Evidence that α2 can be expressed in astrocytes in culture came from mRNA analysis of brain cells grown in culture as reaggregates (Corthesy-Theulaz et al., 1990). The mRNA for all three α isoforms was found in reaggregates after 15 days in culture. The α2 isoform mRNA levels had not yet risen very much after only 7 days in culture, in contrast to α1 and α3 mRNA levels, indicating that accumulation of α2 mRNA was delayed. When reaggregates were treated with cytosine arabinoside at an early time (when the drug eliminates 90 to 97% of glial marker expression by killing proliferating glia) the delayed appearance of α2 mRNA was essentially abolished. In contrast, when neurons were killed by cholera toxin, α2 mRNA levels were markedly increased. The expression of α3 mRNA was unaffected by killing glia, and nearly abolished by killing neurons. Concepts have thus evolved since the early observation of Marks and Seeds (1978): the brain cell reaggregates are now understood to have high-affinity ouabain sites, not only because of the presence of α3 in neurons, but also because of α2 in glia.

Why then is only low-affinity Na,K-ATPase (α1) so often seen in astrocyte monolayers? Recent experiments in my laboratory have shown that, while α1 expression is always seen in cortical glial monolayer cultures, α2 expression is seen only when conditions are permissive for the differentiation or proliferation of process-bearing glial cells. The process-bearing glial cells are not themselves the exclusive locus of the α2, however. Some process-bearing cells express only α1, while flat cells in their vicinity express α2 (K. Sweadner, unpublished observations). The discrepancies in the literature are likely to be due to the plasticity of glia and the variability and heterogeneity of cortical glial cultures. There is abundant precedent that glia in culture are not terminally differentiated. Even the expression of voltage-dependent ion channels is modified by culture environment and cellular interactions (see Chapter 12, this volume); it should come as no surprise that the same is true for an ion pump.

α *Isoform Localization* In Situ

Much has been learned about the cellular distribution of Na,K-ATPase isoforms in the nervous system. There is some evidence that α3 is expressed in many neurons while α2 is expressed in glia, α1 being found in both. This idea originated with the observation of prominent staining for α3 in large-diameter pyramidal neurons, both by immunocytochemistry and *in situ* hybridization (J. Schneider et al., 1988; Brines et al., 1991; McGrail et al., 1991a), while a majority of immunoreactive α2 and α2 mRNA had a diffuse distribution suggestive of glia (Filuk et al., 1989; McGrail and Sweadner, 1989; Anderson et al., 1991; McGrail et al., 1991a). While this generalization has some merit, a closer look at individual cell types, particularly smaller cells and those with less abundant Na,K-ATPase, suggests that isoform expression is more cell type-specific than cell class-specific. It is clear that not all neurons express α3, and not all glia express α1. Neurons have been unambiguously shown to express α1, α2, or α3. The only cell class-specific generalization that has stood up so far is that α3 has never been detected in a glial cell. It is present abundantly, along with α1, in the retinal horizontal cell, however. This is a cell with such unusual characteristics (no synaptic vesicles; extensive intercellular coupling) that it has been considered to be glialike in some respects.

Any given neuronal cell type may express 1 or 2 isoforms simultaneously. For example, α1 and α3

have been detected in retinal ganglion cells, amacrine cells, and horizontal cells (McGrail and Sweadner, 1986, 1989), in hippocampal pyramidal cells (Brines et al., 1991; McGrail et al., 1991a; Pietrini et al., 1992), and in subpopulations of dorsal root ganglion cells (Mata et al., 1991; J. M. Phillips and K. J. Sweadner, unpublished observations). Many large-diameter projection neurons (cortical and cerebellar pyramidal cells for example) express α3 (J. Schneider et al., 1988; Brines et al., 1991; Hieber et al., 1991), while smaller diameter neurons (cerebellar granule cells, for example) express α1 (Brines et al., 1991; Hieber et al., 1991). This also does not stand up as a general rule, however, because of the selective expression of α1, for example, in the large somata of certain brainstem nuclei and cortical strata (Brines et al., 1991; Hieber et al., 1991; McGrail et al., 1991a; Watts et al., 1991), and of α3 in retinal horizontal and bipolar cells (McGrail and Sweadner, 1989). In the dorsal horn of the spinal cord, some large neurons express both α2 and α3 (McGrail et al., 1991a; see also Watts et al., 1991); in the ventral horn, some express both α1 and α3 (Mata et al., 1991). Besides spinal motoneurons, α2 has been found to be expressed in a retinal amacrine cell (McGrail and Sweadner, 1989). Whether α2 is expressed in hippocampal pyramidal cells has been controversial. In two studies of *in situ* hybridization α2 mRNA was seen (Filuk et al., 1989; Brines et al., 1991), while in another it was not (Watts et al., 1991). Immunocytochemical labeling showed strong α2 stain around the cells, but whether the stain included the plasma membrane of the pyramidal cells themselves, as opposed to surrounding glia, was not clear (McGrail et al., 1991a).

The most unambiguous evidence for the presence of α1 and α2 in glia has come from immunocytochemistry. Isolated retinal Müller cells all express α1, but a subpopulation expresses α2 as well (McGrail and Sweadner, 1989). Colocalization of α1 and α2 with GFAP stain (glial fibrillary acidic protein) has been seen in optic nerve (McGrail and Sweadner, 1989) (Figure 16-5). At high magnification, the distinction between the glial localization of α2 and the axonal localization of α3 in spinal cord lateral white matter is striking (McGrail et al., 1991b) (Figure 16-6).

In situ hybridization in glia is usually less distinct than in neuronal somata and more difficult to pinpoint with certainty. At low resolution, α2 and some α1 hybridization has been seen to be diffuse (Filuk et al., 1989; Watts et al., 1991) or localized at low levels in glial-enriched areas (Brines et al., 1991), while α3 and some α1 hybridization is easily seen to be in identifiable neuronal cell bodies. Figure 16-7 shows the clustering of α1, α3, and β1 mRNA in cerebellar Purkinje cell bodies, while α2 and β2 mRNA appear to be located diffusely in adjacent cells, presumably the Bergmann glia (Watts et al., 1991). In the same figure, sensory neurons of the dorsal root ganglion were labeled with α1 and α3 probes, while nonneuronal cells were labeled for α1. Hybridization for α2 was seen in sections of sensory nerve, where only nonneuronal cells have nuclei. Another example is shown in Figure 16-8, where clusters of hybridization for α1 and α3 can be identified in neuronal cell bodies in the superior cervical ganglion, while α2 hybridization is diffuse, as expected for the nonneuronal cells. Schwann cells in sciatic nerve have also been observed to hybridize with probe for α1 mRNA, and white matter tracts of the spinal cord were found to have signal for α1 in glial (presumed oligodendroglial) cell bodies, but not α3 (Mata et al., 1991). One additional striking nonneuronal location of α2 mRNA hybridization is the meninges or arachnoid membrane (Brines et al., 1991; Watts et al., 1991); high-resolution studies have not yet determined whether this labeling is in meningeal cells or closely-apposed astrocytes.

β *Isoforms*

While the Na,K-ATPase α subunit has the multiple-spanning structure expected of an ion transport protein, the β subunit is primarily an extracellular globular protein anchored to the membrane by a single transmembrane segment (Figure 16-4). The localization of the β isoforms has not been as extensively studied as that of the α isoforms, but there is some indication that β2, like α2, may be more commonly expressed in glia. In contrast to β1, β2 mRNA shows a remarkable regional concentration in cerebellar cortex compared to other brain regions (Pagliusi et al., 1990; Anderson et al., 1991). The distribution of β2 there (at low magnification) is similar to that of β1 (Anderson et al., 1991), but at higher magnification β2 mRNA hybridization shows a distribution similar to that of α2 in Bergmann glia, near but not in the neurons (Pagliusi et al., 1990; Watts et al., 1991) (Figure 16-7). Different studies also examined β1 mRNA (Filuk et al., 1989), β2 mRNA (Pagliusi et al., 1990), or both (Watts et al., 1991) in the hippocampal formation. Signal for β1 was highly concentrated in the regions of highest neuronal density, while β2 signal was more distributed, and at high magnification was seen to be associated with glia. A highly specialized neuronal cell, the photoreceptor, expresses β2 but not β1, however, providing evidence that β2 is not an exclusive marker of glia (B. Schneider et al., 1991).

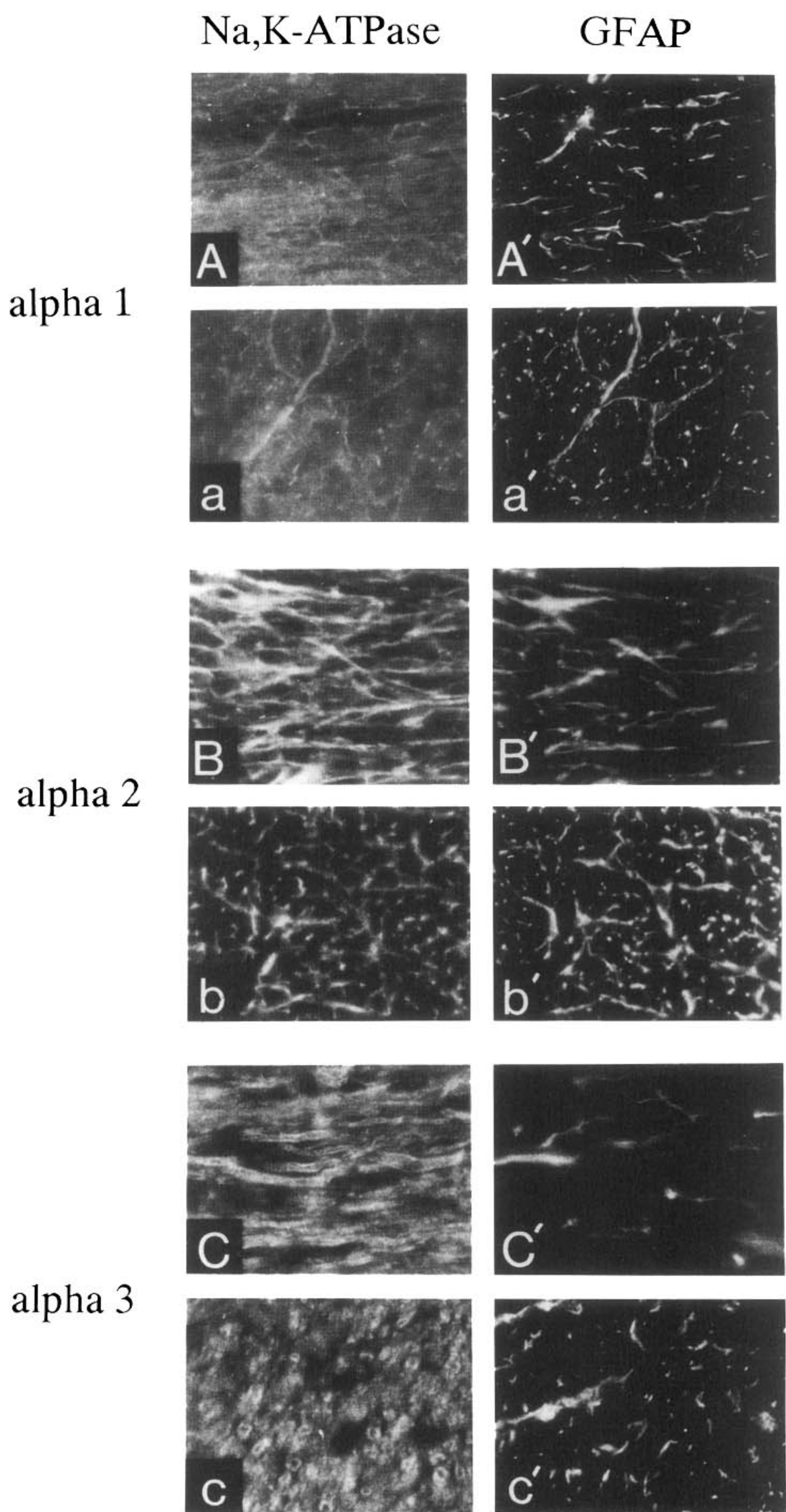
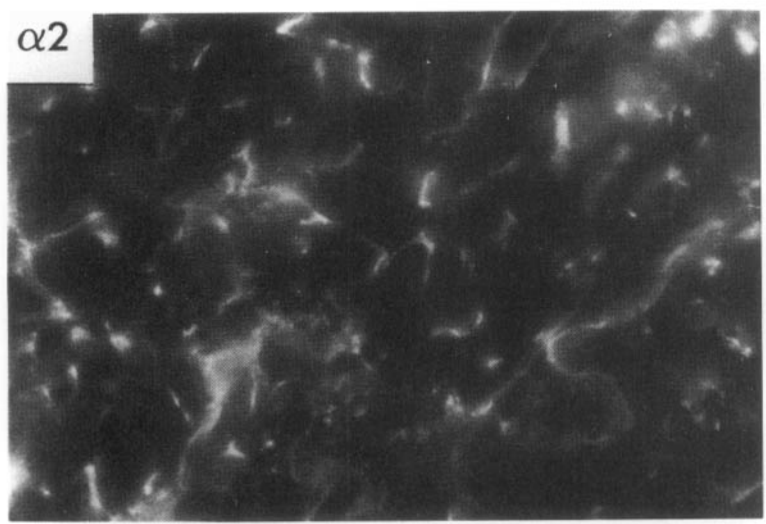


FIG. 16-5. Na,K-ATPase α isoforms in optic nerve. Immunofluorescence was used to stain Na,K-ATPase isoforms. Rat optic nerve was cut in both longitudinal section (*A*, *B*, *C*) and cross section (*a*, *b*, *c*), and double-labeled with monoclonal antibodies (*left*) against $\alpha 1$ (*A*), $\alpha 2$ (*B*), and $\alpha 3$ (*C*) and a rabbit antiserum against a glial marker protein, GFAP (*right*). Both $\alpha 1$ and $\alpha 2$ staining coincided with the glial marker. In contrast, $\alpha 3$ staining outlined the axons. It is notable that the Na,K-ATPase in axons is not confined to nodes of Ranvier. [From McGrail and Sweadner (1989), with permission.]



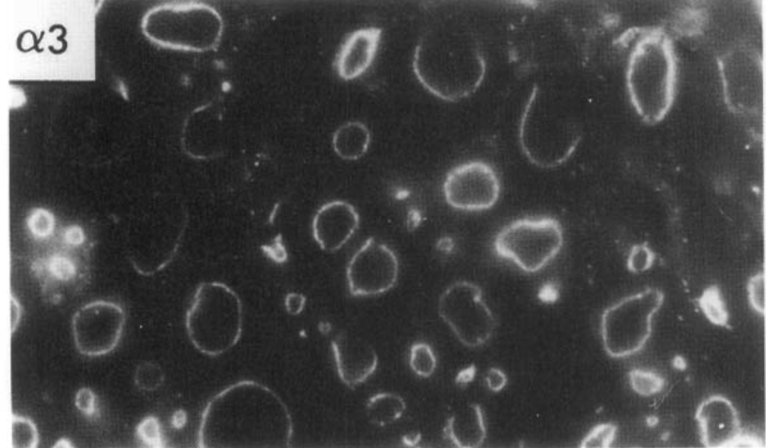


FIG. 16-6. α2 and α3 localization in spinal white matter. Cross sections of white matter adjacent to the dorsal horn were stained with monoclonal antibodies against α2 and α3 by immunofluorescence. The α2 antibody stained glia between the axons, while the α3 antibody ring stained both large- and small-diameter axons. Myelin was unstained, and showed as dark rings surrounding the axons. [From McGrail et al. (1991b), with permission.]

Considerably less immunocytochemistry has been done for the β isoforms because of the lack of a suitable set of antibodies. Staining for β2 has been seen in some (but not all) cultured cortical astrocytes from the mouse, as well as in small cerebellar neurons (Gloor et al., 1990). Recently, however, an antibody originally described to be against a neuron-specific glycoprotein (Beesley et al., 1987) has been found to react with β2 (Gloor et al., 1992; K. Sweadner, unpublished observations).

The cDNA for the β2 subunit was first isolated not as a component of the Na,K-ATPase, but as an adhesion molecule found on cerebellar glia (adhesion molecule on glia [AMOG]) (Pagliusi et al., 1989). Its role in neuron-glia adhesion was documented by the blocking actions of a monoclonal antibody (Antonicek et al., 1987). AMOG (β2) was later shown to be a subunit of the Na,K-ATPase, and to preferentially associate with α2 in detergent extracts of mouse brain (Gloor et al., 1990). It was independently cloned from rat and human brain (Martin-Vasallo et al., 1989) and shown to copurify with Na,K-ATPase α (Shyjan et al., 1990b). The molecular mechanism of its role in adhesion is not yet understood in detail. Two observations make it likely that adhesion mediated by β2 is independent of any structural or functional role it has in the Na,K-ATPase. First, β2 subunit free of α subunit will adhere to neurons when reconstituted into liposomes (Antonicek and Schachner, 1988), and second, AMOG-mediated neuron-glia adhesion can occur even when the Na,K-ATPase is inhibited by ouabain (Gloor et al., 1990). The glycosylated β2 may be the recognition target of a more conventional adhesion protein; it shares the L3 carbohydrate epitope with other adhesion proteins L1 and MAG (Gloor et al., 1990).

FUNCTIONAL DIFFERENCES BETWEEN Na,K-ATPase ISOFORMS

A question of considerable importance is how the isoforms differ functionally, and whether these dif-

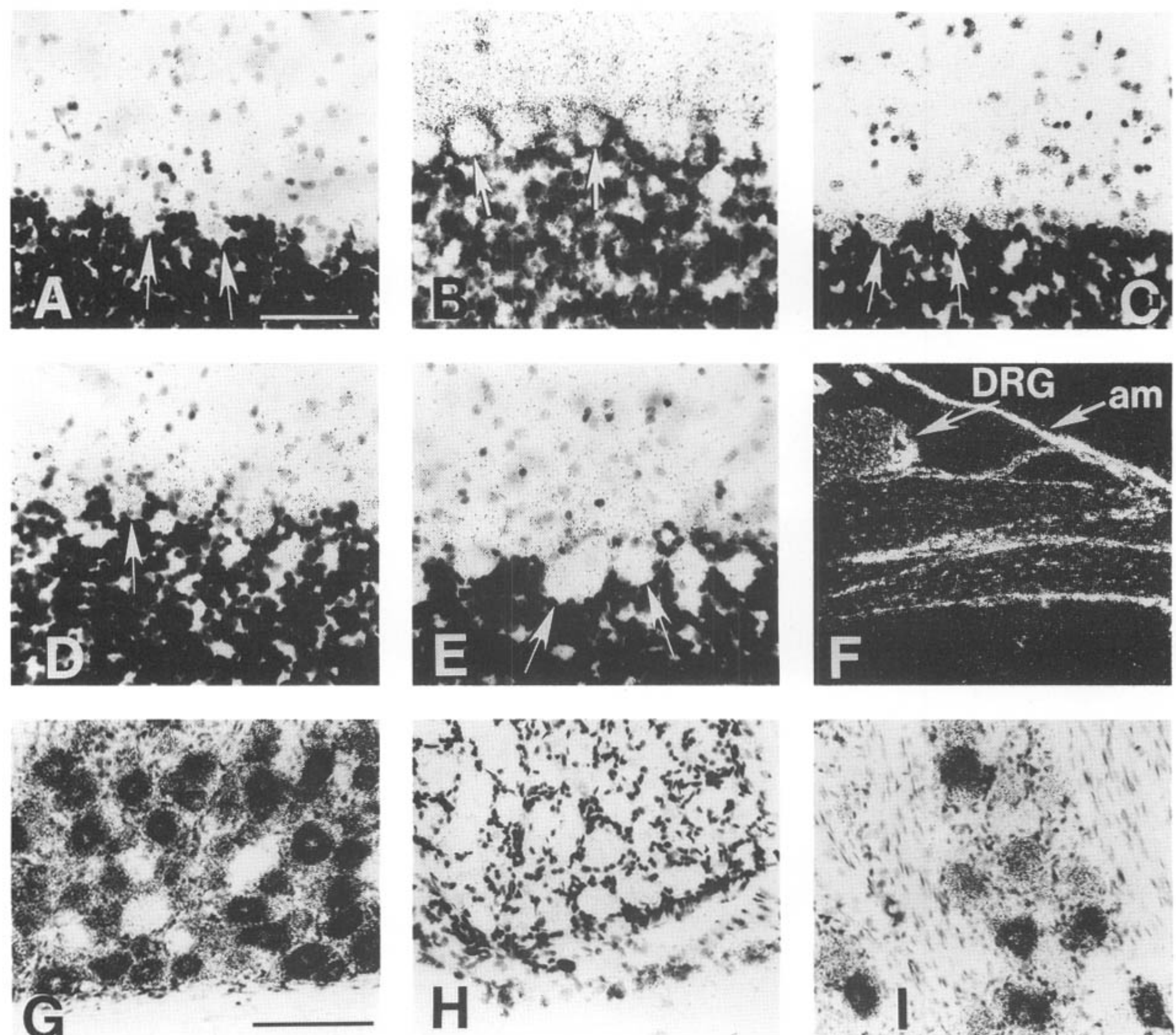


FIG. 16-7. *In situ* hybridization in cerebellum and dorsal root ganglion. Brightfield photographs of hybridization for $\alpha 1$ (*A*), $\alpha 2$ (*B*), $\alpha 3$ (*C*), $\beta 1$ (*D*), and $\beta 2$ (*E*) in sections of the adult rat cerebellum. Purkinje cell bodies are indicated with *arrows*. The hybridization signal is the tiny dark grains. The cell bodies of the granule cell layer are stained with thionin, and thus are dark, obscuring any hybridization. Darkfield image of $\alpha 2$ hybridization in dorsal root ganglion (*F*); am, arachnoid membrane. Brightfield photographs of $\alpha 1$ (*G*), $\alpha 2$ (*H*), and $\alpha 3$ (*I*) hybridization in dorsal root ganglion. [From Watts et al. (1991), with permission.]

ferences play a role in glial physiology. A few things are known about their substrate affinities and possible selective regulation by hormones, although there is much more controversy than consensus.

Possible differences in affinity for Na^+ have received the most attention, although a clear picture has not yet emerged. Affinity for Na^+ for axolemma Na,K-ATPase preparations containing α2 and α3 has been reported to be higher than in kidney preparations containing α1 (Sweadner, 1985). Several enzymological criteria suggest that this may be related to the tendency of α1 to adopt the E2 conformation more than α2 + α3 (reviewed in Sweadner, 1989). When the Na^+ affinity of pinealocyte membrane Na,K-ATPase, containing α3, was compared to that of kidney Na,K-ATPase (α1), it was found that the α3 had a higher affinity (Shyjan et al., 1990a), although both measured affinities (11 mM and 20 mM, respectively) were substantially lower than reported for axolemma and kidney at the same concentration of K^+ (6 mM and 10 mM) (Sweadner, 1985). When α1, α2, and α3 were expressed separately in transfected HeLa cells, α1 and α2 were found to have similar affinities ($K_{0.5}$ about 5 mM) while α3 was of lower affinity ($K_{0.5}$ about 12 mM) (Jewell and Lingrel, 1991). That α1 and α2 have essentially the same Na^+ affinities was determined independently in transfected CV-1 cells with a dif-

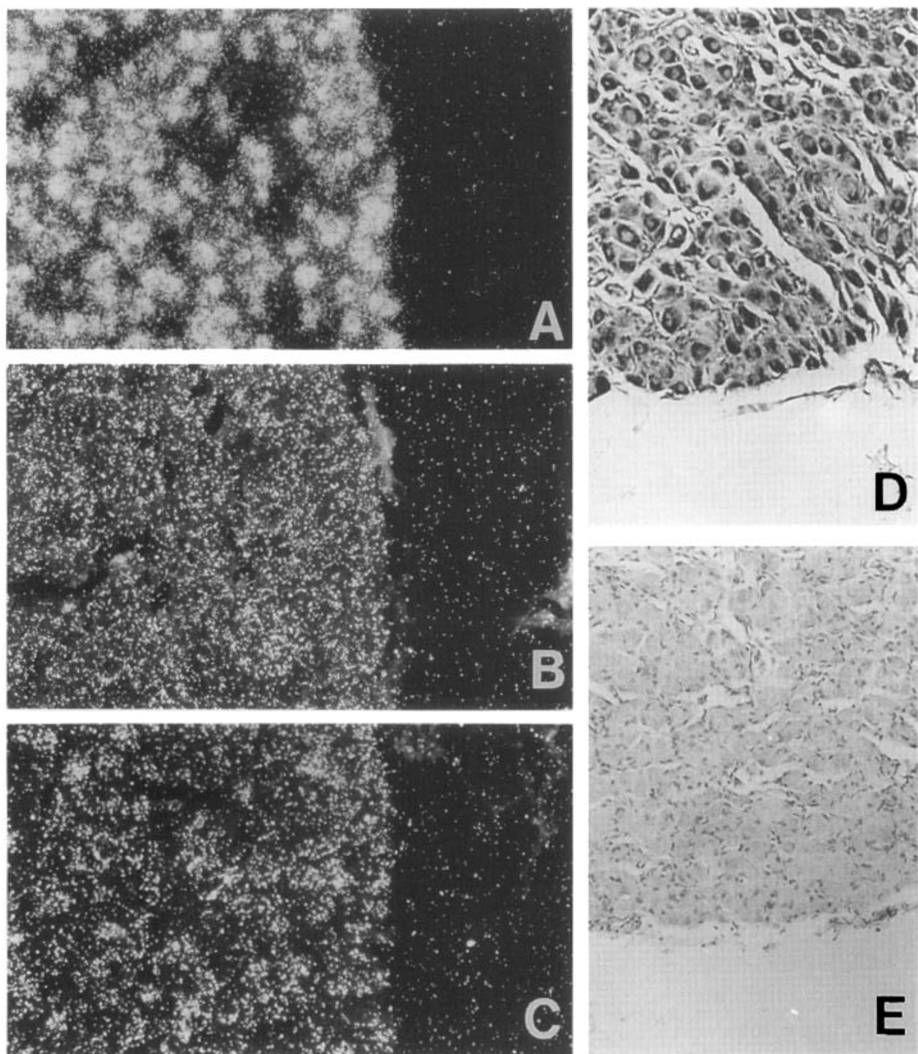


FIG. 16-8. Darkfield photographs of hybridization for $\alpha 1$ (*A*), $\alpha 2$ (*B*), or $\alpha 3$ (*C*) are shown; the signal is the white spots. Hybridization signal for $\alpha 1$ was heavily concentrated in neuronal cell bodies, and $\alpha 3$ was concentrated in a subset of them. Hybridization for $\alpha 2$, in contrast, was very diffuse. *In situ* hybridization for α isoforms in superior cervical ganglion. Sections of adult rat superior cervical ganglion (a ganglion of the sympathetic nervous system) were stained with hematoxylin/eosin (*D*) to highlight the neuronal cell bodies or with methylene blue (*E*) to highlight the non-neuronal cell nuclei. [T. Cova, K. Sweadner, and V. H. M. Herrera, unpublished observations.]

ferent assay: $^{86}Rb^+$ uptake in Na^+-loaded cells (Shyjan et al., 1991). The affinity of α3 in transfected HeLa cells (Jewell and Lingrel, 1991) was very similar to that of α3 in pineal cells (Shyjan et al., 1990a), but α3 Na^+ affinities were lower than measured in axolemma, and kidney α1 affinity measurements have not been very reproducible from laboratory to laboratory. Jewell and Lingrel (1993) made chimeras of α1 and α3 to attempt to pinpoint regions essential for the difference in Na^+ affinity in the sequence of the α subunit. The outcome was that no single region determined Na^+ affinity. Such disparate conclusions suggest that Na^+ affinity is not an unchangeable property of each Na,K-ATPase isoform, but rather that it is something modified by regulation or environment, or even by association with different β subunits.

Alteration of Na,K-ATPase Na^+ affinity by insulin has in fact been extensively investigated by Guidotti and collaborators. Adipocyte α2 was originally thought to be of low Na^+ affinity unless treated with insulin (Lytton, 1985), but when this was reinvestigated in adipocyte ghosts it was shown that α1 and α2 affinities were really very similar, and changes in intracellular Na^+ concentration had given a deceptively high value for the relative level of activity of the α1 isoform (McGill, 1991). Simulations of the effect of inhibiting one isoform while raising intracellular Na^+ allowed a calculation of the effect of insulin on both K_m and V_{max} (McGill and Guidotti, 1991): it was concluded that the K_m for both α1 and α2 could be affected by insulin, but that the major physiological effect was more likely on the V_{max} for α2. Similar phenomena were reported in synaptosomes, containing all three isoforms of the Na,K-ATPase (Brodsky, 1990; Brodsky and Guidotti, 1990). The mechanisms by which insulin exerts its effects are still unknown.

Potassium affinity is of particular interest because of its potential role in glial physiology, but again, no

consistent pattern has been seen when Na,K-ATPase isoforms have been compared. In transfected HeLa cells, α1 and α2 had indistinguishable affinities of 0.45 mM, while α3 had a higher affinity, 0.27 mM, measured in 30 mM NaCl (Jewell and Lingrel, 1991). When kidney (α1) and pinealocyte (α3) membranes were compared, α1 had an affinity of 1.75 mM, while α3 had an affinity of 2.1 mM, measured in 130 mM Na^+ (Shyjan et al., 1990a). In purified enzyme preparations from kidney (α1) and axolemma (α2 + α3), K^+ affinities were essentially indistinguishable (0.88 mM and 0.91 mM), measured in 140 mM Na^+ (Sweadner, 1985). Recently, however, evidence has been presented that the isoform of the β subunit affects the K^+ affinity of the Na,K-ATPase (Jaisser et al., 1992). While β1 is the only isoform present in the HeLa cells and kidney preparations, β2 predominates in pinealocytes, and both may be present in axolemma.

SUMMARY

The role of the Na,K-ATPase in glia is still surprisingly controversial. The discovery of molecular heterogeneity of its subunits has been a big step forward, but detailed cellular physiology and biophysical studies are needed to answer some of the major questions, such as whether and how much the pump contributes to K^+ redistribution, and why astrocytes express more than one isoform of both α and β subunits. The question of whether astrocyte Na,K-ATPase has a low affinity for K^+, making it suitable to play a role in K^+ clearance, remains controversial.

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17 Modulation and control of intracellular calcium

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The importance of understanding glial calcium (Ca^{2+}) control seems clear: the calcium ion modulates or mediates many cellular functions and may underlie a form of long-range signaling (Finkbeiner, 1993). Transient cytosolic calcium changes produce several immediate, intermediate, and long-term changes in glial structure and function. Within seconds of a calcium increase, glial calcium-activated potassium channels open, transiently changing the composition of the interstitial space (Barres et al., 1990). Calcium increases may quickly mobilize arachidonic acid, which in turn may alter extracellular glutamate levels (Marin et al., 1991b; El-Etr et al., 1992). Calcium-activated protein kinases such as calcium/calmodulin-dependent kinase (Fukunaga et al., 1988; Schulman et al., 1992) and phospholipid-dependent protein kinase C (PKC) (Neary et al., 1986; Murphy et al., 1993) translate transient calcium changes into longer-lasting functional changes through phosphorylation of enzymes, ion channels, and the cytoskeleton (Huganir, 1987; Harrison and Mobley, 1992; Medrano et al., 1992). Calcium increases produce long-term changes by modulating proteolytic cytoskeletal remodeling (Schlaepfer and Zimmerman, 1981; Bianchi et al., 1993) and glial gene expression (Trejo and Brown, 1991; Bagaglio et al., 1993), ultimately affecting glial proliferation (Meador-Woodruff et al., 1984; Supattapone et al., 1989) and differentiation (Hart et al., 1989). This chapter focuses on the cell biology and physiology of glial calcium, including its intracellular distribution, control mechanisms, and the temporal and spatial patterns of stimulus-evoked calcium change. Chapter 12 and Section IV discuss glial voltage-gated calcium channels and neurotransmitter receptors.

CALCIUM DISTRIBUTION AND HOMEOSTASIS

Cells distribute calcium among three functional compartments: extracellular space, cytosol, and restricted intracellular sites known as calcium stores or compartments. Normally, glia maintain an asymmetric distribution of calcium. Free cytosolic calcium is low (100 nM), extracellular calcium is high (2 to 5 mM), and the calcium content of intracellular stores can be very high (1 to 100 mM). Stimulation may evoke transmembrane influx of extracellular calcium or release of internally stored calcium sufficient to raise cytoplasmic levels to 1 to 2 μM (Finkbeiner, 1993).

Glia restore and maintain baseline calcium distribution by pumping calcium from the cytosol into internal stores or across the plasma membrane. Two main types of calcium pump, sodium-calcium exchangers and calcium-adenosine triphosphatases (ATPases), shuttle calcium between compartments in other cell types (Muallem, 1989). No direct proof exists for glial sodium-calcium exchange but several results suggest it occurs. The sodium-calcium exchanger depends on a transmembrane sodium gradient to extrude intracellular calcium. Sodium-free solution itself raises glial cytoplasmic calcium (Cornell-Bell and Finkbeiner, 1991; Delumeau et al., 1991; MacVicar et al., 1991) and alters the magnitude and shape of glial calcium responses (Cornell-Bell et al., 1990; Finkbeiner, 1991) (Figure 17-1). Calcium/ATPase localizes to glial plasma membranes (Mata et al., 1988) and endoplasmic reticulum (Maggio et al., 1991) and is active in central and peripheral nervous system glia (Mata and Fink, 1989). A calcium-hydrogen exchanger may pump calcium into internal stores of certain invertebrate glial cells (Keicher et al., 1991).

INTRACELLULAR CALCIUM STORES

According to one radiotracer study, the calcium content of glial intracellular stores exceeds free cytosolic calcium by a ratio of 20,000:1 (Walz and Wilson, 1986). The calcium is distributed among several intracellular structures, including cytoplasmic calcium-binding proteins, mitochondria, lysozomes, the Golgi apparatus, and elements of the endoplasmic reticulum; the endoplasmic reticulum-like store contains more calcium than the other organelles (Gambetti et

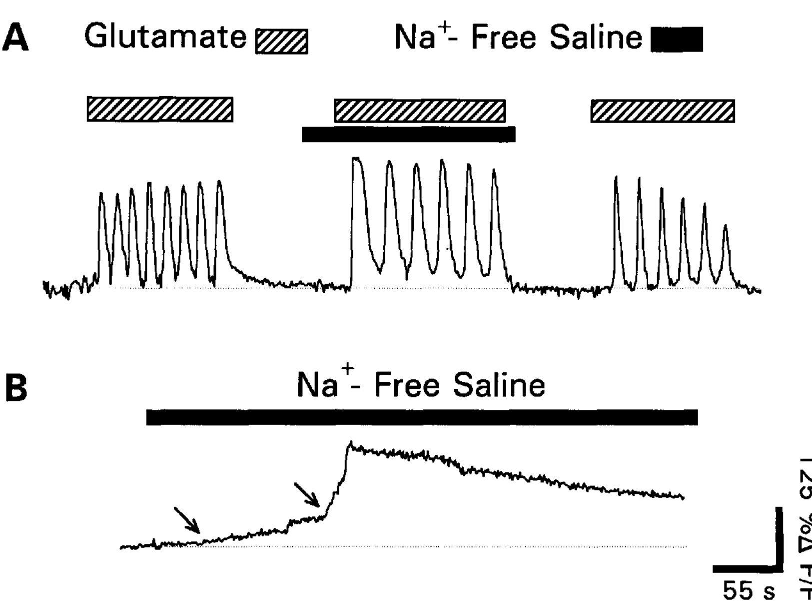

FIG. 17-1. Na^+-free saline application evokes glial Ca^{2+} increases and enhances stimulus-evoked Ca^{2+} responses. The figure shows fluo-3 fluorescence time-course traces from rat hippocampal type-1 astrocytes (Cornell-Bell et al., 1990; Finkbeiner, 1991). Na^+-free saline was prepared by isosmotic Na^+ substitution with impermeable *N*-methyl-D-glucamine or choline. The addition bars indicate the duration of glutamate or Na^+-free saline application. (A) Na^+-free saline increases the amplitude and duration of glutamate-evoked cytosolic Ca^{2+} oscillations. (B) The change to Na^+-free superfusate also marks the beginning of a slow steady rise in cytoplasmic Ca^{2+} (*first arrow*). Three minutes after Na^+-free saline washes on, the astrocyte spontaneously initiates a propagating Ca^{2+} wave, indicated by the abrupt Ca^{2+} rise (*second arrow*). Typically, ligand-induced Ca^{2+} increases have a spike and plateau shape (*see text*). However, Ca^{2+} increases in Na^+-free saline remain near peak levels for long periods and return toward baseline slowly.

al., 1975; Vidulescu et al., 1991). The location of calcium stores differs among glia types. In spinal cord ependymal cells, the calcium-containing endoplasmic reticulum elements have a vesicular-cisternal morphology and associate with cytoskeletal elements in cell processes (Gambetti et al., 1975). In central nervous system astrocytes, the analogous structures have a circular shape and appear throughout the cytoplasm. In hippocampal astrocytes, the perinuclear endoplasmic reticulum elements are particularly rich in calcium (Cornell-Bell and Finkbeiner, 1991). Calreticulin, a low-affinity, high-capacity calcium-binding protein, is probably present in glial endoplasmic reticulum and enables the organelle to store large amounts of calcium (Lytton and Nigam, 1992; Opas and Michalak, 1992). Certain invertebrate glia have calcium containing (50 to 100 mM) acidic organelles known as gliagrana that localize to glial processes extending into the perineuronal space (Keicher et al., 1991).

Certain glial calcium-containing organelles likely participate in intracellular signaling by undergoing stimulus-evoked calcium release. In other cell types, endoplasmic reticulum components mediate this function (Terasaki and Sardet, 1991). Glutamate, a neurotransmitter that releases internally stored calcium in astrocytes, shifts histochemical calcium staining to the cytoplasm from endoplasmic reticulum-like structures, mitochondria, and the nucleus (Cornell-Bell and Finkbeiner, 1991). Although mitochondria represent a large and dynamic internal calcium store, it is unlikely that they directly undergo ligand-induced calcium release (Zareba-Kowalska and Gajkowska, 1990; Gill et al., 1992).

The functional organization and biochemical control of intracellular calcium stores is a subject of intense study (for review see Berridge, 1993). There are at least two mechanisms by which internal stores release calcium. In one mechanism, a ligand binds to a surface receptor that activates phospholipase C (PLC) through a G protein or through a tyrosine kinase. Phospholipase C metabolizes phosphatidylinositol 4,5-bisphosphate to form inositol 1,4,5-trisphosphate (IP_3), which diffuses from the plasma membrane through the cytosol to activate a calcium channel located on intracellular stores (Terasaki and Sardet, 1991; Taylor and Marshall, 1992). Several lines of evidence suggest that this pathway is present in glia.

1. Most ligands capable of intracellular Ca^{2+} release also stimulate glial IP_3 production (Murphy and Pearce, 1987; Pearce et al., 1988).
2. Blocking intracellular Ca^{2+} release with 8-(*N,N*-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), dramatically decreases Ca^{2+} responses to some IP_3-mobilizing ligands (Lin et al., 1992).
3. Some ligands that stimulate inositol turnover release Ca^{2+} from the same intracellular Ca^{2+} stores (McDonough et al., 1988; Lin et al., 1992).
4. Pertussis toxin (PTX), a protein that inactivates a subset of G proteins, diminishes glial Ca^{2+} responses to a few of the inositol-mobilizing ligands (Delumeau et al., 1991; Marin et al., 1991b).

5. Nanomolar concentrations of IP_3 produced by flash photolysis evoke rapid cytoplasmic Ca^{2+} increases in cerebellar astrocytes (Khodakhah and Ogden, 1993).
6. Inhibition of Ca^{2+}/ATPase, the enzyme that pumps Ca^{2+} into most internal stores, eliminates glutamate Ca^{2+} responses in astrocytes (Charles et al., 1993).

A second mechanism to release internal calcium is through calcium-induced calcium release (CICR) (Putney, 1986). Skeletal and cardiac muscle cells utilize CICR as the major mechanism to achieve excitation-induced cytoplasmic calcium increases. The calcium ion binds an IP_3 receptor homologue called the *ryanodine receptor*, which gates a sarcoplasmic reticulum calcium channel (for review see Taylor and Marshall, 1992). Glia express plasma membrane calcium-activated calcium channels; however, it is unclear whether such a channel regulates release of internal calcium stores (Puro, 1991b). In fact, ryanodine failed to elicit calcium responses itself or modulate glial calcium responses to glutamate or mechanical stimulation (Finkbeiner, 1991; Charles et al., 1993). Caffeine, which triggers CICR in other cell types, was similarly ineffective on cortical astrocytes (Charles et al., 1993).

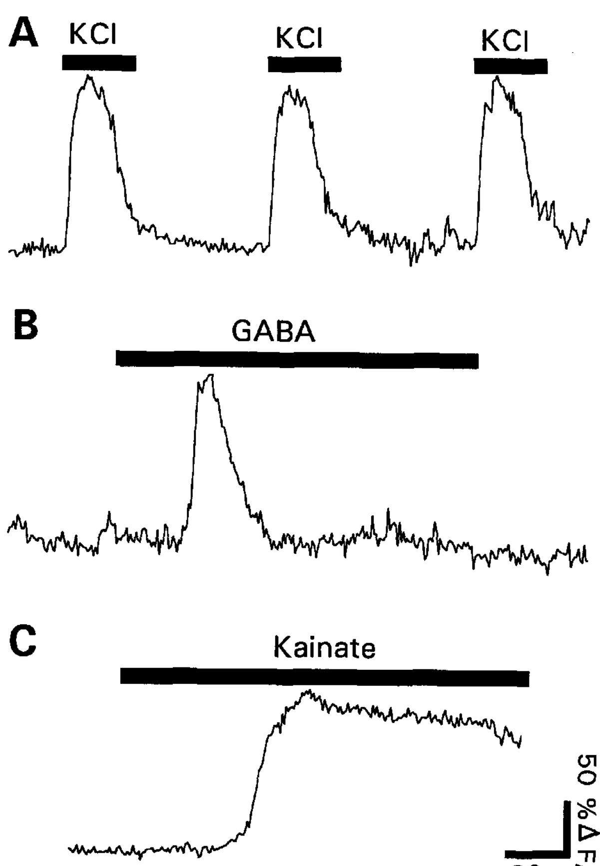

FIG. 17-2. Monophasic Ca^{2+} responses illustrated with fluorescence time-course traces from fluo-3 experiments on rat hippocampal type-1 astrocytes (Finkbeiner, 1991). (A) Ca^{2+} rises and falls quickly in response to depolarizing K^+ (55 mM) pulses (addition bars). Dihydropyridine (DHP)-sensitive voltage-gated Ca^{2+} channels likely mediate the rise as the removal of extracellular Ca^{2+} or the addition of the DHP, nimodipine blocks the K^+-induced Ca^{2+} rise (Finkbeiner, 1991). (B) GABA (100 μM) Ca^{2+} responses appear long after GABA washes on and disappear well before GABA washes off. (C) Kainate (100 μM) stimulates a cytoplasmic Ca^{2+} response, which gradually rises to a maximum then gradually declines to baseline, despite the continued presence of kainate.

PLASMA MEMBRANE CALCIUM CHANNELS

Voltage-Gated Calcium Channels

Glial cells possess several calcium-permeable plasma membrane ion channels. Sontheimer and Ritchie review glial voltage-gated calcium channels in Chapter 12, but a few points are particularly relevant to this discussion. Glia express several channels that differ in the membrane potential at which they activate, the amount of calcium conducted per single channel opening, and the rate and control of channel inactivation (Sontheimer, 1992). Channel activation triggers sufficient calcium influx to significantly raise glial cytoplasmic calcium levels (Jensen and Chiu, 1990, 1991; Finkbeiner, 1991; Fatatis and Russell, 1992; Munsch and Deitmer, 1992) (Figure 17-2). Finally, glial calcium channel expression depends on environmental factors and may be under neuronal control *in vivo* (Corvalan et al., 1990; MacVicar et al., 1991; Matute and Miledi, 1993).

Ligand-Gated Calcium Channels

Another heterogeneous group of glial plasma membrane calcium channels are not voltage-activated. Rather these channels open in response to certain ligands, cytoplasmic calcium increases, or deformation of the plasma membrane. For example, the glutamate analogues kainate or α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA) activate receptor-gated calcium channels in Bergmann glia. Simultaneous electrophysiologic and fluorometric measurements show that activation of AMPA/kainate-sensitive glutamate receptors produces significant calcium influx independent of voltage changes (Müller et al., 1992). Quantitatively, calcium permeates the channel 1.5 times the rate of the monovalent ion, cesium. Bergmann glia express a particular subset of glutamate receptor subunits, which confer calcium permeability to the channels they comprise (Burnashev et al., 1992).

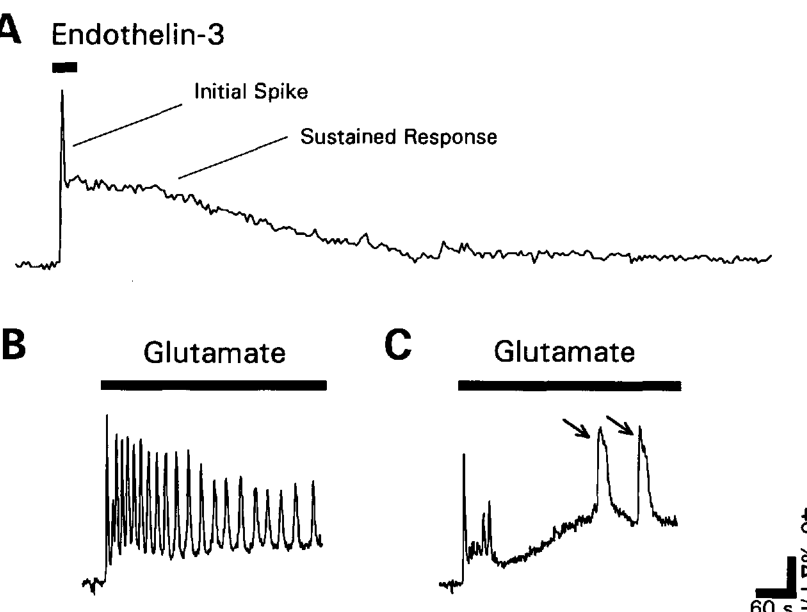

FIG. 17-3. Biphasic/polyphasic Ca^{2+} responses from fluo-3 experiments on rat hippocampal type-1 astrocytes (Finkbeiner, 1991). (A) The small peptide, endothelin-3, stimulates an initial Ca^{2+} spike lasting seconds and a sustained plateau lasting 20 to 30 minutes. The two components are labeled. The sustained component of glutamate Ca^{2+} responses (B) may manifest complex cytosolic Ca^{2+} oscillations or (C) may be interrupted by episodic broad-peaked Ca^{+} rises (*arrows*), which represent intracellular and intercellular Ca^{2+} waves.

Most studies find that another glutamate receptor subtype, the *N*-methyl-D-aspartate (NMDA) receptor, does not mediate glial calcium responses (Enkvist et al., 1989b; Glaum et al., 1990; Jensen and Chiu, 1990). However, two studies differ. In the study by Ahmed et al. (1990), a small minority of spinal cord astrocytes show NMDA-induced calcium increases in the presence of extracellular calcium. In the study by Holopainen and Åkerman (1990), NMDA induced a calcium 45 efflux from cultured cerebral astrocytes, which changed predictably upon application or withdrawal of NMDA antagonists. Recently, one group found high doses of NMDA elicited small non-magnesium-sensitive, non-glycine-sensitive inward currents in Bergmann glia (Müller et al., 1993). However, the NMDA responses did not produce detectable calcium rises. Perhaps the calcium responses detected in the earlier studies resulted from activation of voltage-gated calcium fluxes by NMDA-induced depolarization.

Some researchers suggest benzodiazepine and kappa-opiod receptors directly activate voltage-gated calcium channels. In support, benzodiazepines competitively inhibit the binding of nitrendipine, a dihydropyridine (DHP)-sensitive calcium channel blocker (Bender and Hertz, 1985). However, direct electrophysiological evidence is lacking, and follow-up data have raised doubts about the hypothesis (Pruss et al., 1991). Kappa-opiod receptor agonists trigger dihydropyridine-sensitive voltage-gated calcium fluxes, although the transduction mechanism is unclear (Eriksson et al., 1993).

Retinal Müller glia express two interesting calcium channels: one opens in response to cytosolic calcium increases, the other in response to plasma membrane deformation. The calcium-activated calcium channel is detectable in 50% of cells and has an average single-channel conductance of approximately 17 pS (Puro, 1991b). Cytosolic calcium concentrations govern the fraction of time in which the channel remains in the open state: single-channel openings increase 10-fold when calcium applied to the cytoplasmic side of excised patches increases from 10 nM to 10 μM. The second channel type, a "stretch-activated" ion channel, is permeable to both monovalent and divalent cations and has a single-channel conductance of 32 pS (Puro, 1991a). Channel activation produces sufficient calcium influx to increase calcium-activated potassium currents.

STIMULUS-EVOKED GLIAL CALCIUM SIGNALING

Glia show remarkably diverse and complex calcium responses to a variety of stimuli, including neurotransmitters, neuropeptides, neuromodulators, second messengers, depolarization, and mechanical stimulation (Finkbeiner, 1993) (Figures 17-2 and 17-3; Table 17-1). The shape of the calcium fluctuation ranges from a simple step-response to a complex oscillation: the latency and duration of responses vary too. Calcium responses may be restricted to single glial cells or propagate between cells. The complexity and diversity of glial calcium responses raise several intriguing questions. What glial signal transduction pathways must exist to produce such diversity? How do glial cells achieve sig-

TABLE 17-1. *Stimulus-Evoked Glial Ca^{2+} Responses*

Stimulus/ Effector or Receptor/ Subtype	Intracellular Release	Transmembrane Influx	Predominant Ca^{2+} Response Pattern			
					Polyphasic	
			Monophasic	Biphasic	Oscillations	Waves
Adrenergic receptor						
α_1	+++	+	+	−	+	+
α_2	?	?	+	+	?	?
β_1	+/−	+	+	+	+	−
Angiotensin II	++	?	+	+	?	?
Arachidonic acid	+	+++	+	−	−	−
Bradykinin						
B_2	+/−	++	+	+	+	+
Cholinergic receptor						
Muscarinic	+++	+	−	+	+	−
Depolarization (KCl 55 mM)	+/−	++	+	−	−	−
Endothelin	+++	++	−	+	+	−
GABA						
$GABA_A$	++	+	+	+	−	−
$GABA_B$	−	++	+	−	−	−
Glutamate	+++	++	+	+	+	+
ACPD	++	−	−	+	+	−
AMPA	+	++	+	+	+	?
Kainate	+/−	++	+	−	−	+
Quisqualate	+++	+	−	+	+	+
Histamine						
H_1	++	++	−	+	+	+
Opioid						
Kappa	−	++	+	−	−	−
Oxytocin	++	+/−	−	+	?	?
Pancreatic polypeptide						
NPY	−	++	+	−	−	−
Purinergic receptor						
P_1	?	++	?	?	?	?
A_1	++	+/−	−	+	−	−
P_2	+++	++	+	+	+	+
P_{2Y}	+++	++	−	+	?	?
Serotonin						
$5\text{-}HT_2$	+++	+	−	+	+	+
Tachykinin						
NK-1	+++	+	+	+	+	−
Thrombin	?	?	+	−	?	?
Vasopressin						
V_1	+++	+	−	+	?	?

+, present; −, absent; ?, underdetermined.
GABA, gamma-aminobutyric acid; ACPD, *trans*-1-aminocyclopentane-1,3-dicarboxylic acid; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; NPY, neuropeptide.

naling specificity when different stimuli produce similar responses or a single stimulus produces diverse responses? Do these responses, particularly long-range glial signaling, occur *in vivo* and what purpose do they serve?

Response Classification

Glial calcium responses can be broadly classified as either monophasic or biphasic/polyphasic (Finkbeiner, 1993). Stimuli such as kainate, the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), or depolarizing concentrations of potassium typically evoke simple monophasic responses (Figure 17-2). In monophasic responses, cytoplasmic calcium steadily climbs to a peak, then it may or may not decline to baseline: the rate of rise, plateau duration, and rate of return to baseline vary. By contrast, stimuli such as glutamate or the neuropeptide endothelin (ET) produce complex biphasic or polyphasic responses (Figure 17-3). In biphasic/polyphasic responses, calcium rises rapidly to form an initial spike, then falls to an intermediate level for an indefinite interval. The sustained calcium rise following the initial spike may manifest different patterns, including periodic calcium oscillations, large random calcium peaks, or a flat, featureless calcium plateau.

Calcium Source for Cytosolic Transients

The importance of the response categories partly relates to the mechanism and source of the calcium increase. Monophasic responses usually result from transmembrane calcium influx through ligand- or voltage-gated plasma membrane calcium channels: biphasic/polyphasic responses depend on second messenger-induced internal calcium release and variable amounts of calcium influx through plasma membrane calcium channels (Table 17-1). To test whether a stimulus-evoked response depends on influx of extracellular calcium, release of internal calcium stores, or both, one compares the response in the presence and absence of extracellular calcium. Extracellular calcium removal nearly eliminates calcium responses to some stimuli, such as depolarizing potassium (Finkbeiner, 1991; Munsch and Deitmer, 1992; van den Pol et al., 1992), but has no effect on responses to other stimuli, such as the inositol-mobilizing glutamate analogue, *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) (Cornell-Bell and Finkbeiner, 1991). The results are consistent with the idea that depolarization causes calcium influx through voltage-gated calcium channels, and ACPD releases internal calcium through IP_3.

Biphasic/polyphasic calcium responses depend on internal calcium release as well as extracellular calcium influx. The initial calcium spike primarily results from intracellular store release. In support, the spike usually persists in the absence of extracellular calcium. The nucleotide adenosine triphosphate (ATP), the glutamate analogues quisqualate and ACPD, the neurotransmitters histamine, acetylcholine (ACh), and norepinephrine, or the neuropeptides bradykinin and neuropeptide Y (NPY) each produce a calcium spike in calcium-free saline (Brooks et al., 1989; Ahmed et al., 1990; Cornell-Bell et al., 1990; Glaum et al., 1990; Cornell-Bell and Finkbeiner, 1991; Fukui et al., 1991; Neary et al., 1991; Nilsson et al., 1991; Gimpl et al., 1992, 1993). For some stimuli, a portion of the initial spike may depend on calcium influx (Jensen and Chiu, 1990; Marin et al., 1991a; Kastritsis et al., 1992).

By contrast, continued calcium influx is necessary to sustain biphasic/polyphasic responses. Removal of extracellular calcium eliminates the sustained component of responses evoked by acetylcholine (Brooks et al., 1989), ATP (Neary et al., 1991; Kastritsis et al., 1992), the A_1-purinergic receptor agonist 2-chloroadenosine (2-CA) (El-Etr et al., 1992), endothelin (Goldman et al., 1991; Marin et al., 1991a), histamine (Fukui et al., 1991), the α_1-adrenoreceptor agonist methoxamine (Iuvone et al., 1991), norepinephrine (Salm and McCarthy, 1990; Iuvone et al., 1991) or quisqualate (Glaum et al., 1990; Jensen and Chiu, 1990). In other cell types the sustained response follows internal calcium release and results from influx through nickel-sensitive, dihydropyridine-insensitive, voltage-independent plasma membrane channels (Penner et al., 1988). Since these channels open in response to empty internal calcium stores they are also called calcium release-activated channels (CRAC): CRACs are different from the calcium-activated calcium channels gated by the ryanodine receptor (Penner et al., 1993). Endothelin triggers sustained cytoplasmic calcium rises in astrocytes by tonically increasing plasma membrane calcium permeability consistent with CRAC activation (Marin et al., 1991a). Quisqualate (Glaum et al., 1990) and histamine (Fukui et al., 1991) induce glial calcium responses with similar features. However, internal store release may not always lead to a detectable sustained calcium influx. Low doses of endothelin or norepinephrine evoke an initial calcium spike without a subsequent sustained component (Salm and McCarthy, 1990; Goldman et al., 1991). In one experiment, phorbol ester exposure selectively blocked the internal release component but not the prolonged influx and the persistent plateau of acetylcholine-evoked calcium responses (Brooks et al., 1989).

In some polyphasic responses, the calcium level of the sustained component fluctuates in an oscillatory pattern (Figure 17-3B). Stimulus-evoked calcium oscillations persist in the absence of extracellular calcium, although the oscillations are usually less numerous, have smaller amplitudes (Charles et al., 1991; Kastritsis et al., 1992), and occur in a smaller fraction of cells (Cornell-Bell et al., 1990; Salm and McCarthy, 1990; Jensen and Chiu, 1991). Application of the inorganic calcium channel blockers nickel (Glaum et al., 1990; Jensen and Chiu, 1990; Marin et al., 1991a) or lanthanum (Fukui et al., 1991; Neary et al., 1991) also inhibit sustained calcium increases. Together, these results suggest that tonic calcium influx sustains the oscillation mechanism, although the oscillations themselves do not represent periodic calcium influx. The literature is replete with biochemical models of calcium oscillations; the details are beyond the scope of this chapter (for an excellent review see Fewtrell, 1993). Recent experiments suggest that the calcium ion itself can enhance or inhibit IP_3-activated release of internal calcium stores in a concentration dependent manner: the interaction may underlie the bistability necessary to generate calcium oscillations (Yao and Parker, 1992; Dupont and Goldbeter, 1993).

TEMPORAL ASPECTS OF GLIAL CALCIUM SIGNALING

Response Latency and Duration

The latency and duration of stimulus-coupled calcium responses vary considerably. Stimuli such as depolarizing potassium raise cytosolic calcium to maximal levels within seconds of application; calcium rapidly returns to baseline upon potassium removal (Figure 17-2A). Other stimuli such as GABA or kainate may show a significant delay between application and calcium response (Figures 17-2B, C) (Finkbeiner, 1991). Kainate- or GABA-induced calcium rises also grow more slowly, requiring several seconds to achieve peak levels (Dave et al., 1991; Finkbeiner, 1991). By contrast, glutamate or endothelin produces a rapid spikelike calcium rise, which peaks within a second or two of stimulation (Figure 17-3). Many GABA calcium responses ceased before agonist removal, whereas seconds of endothelin application raised cytosolic calcium for 20 to 30 minutes (Supattapone et al., 1989; Marin et al., 1991a) (Figures 17-2B and 17-3A). Cortical type-1 astrocytes similarly show GABA calcium responses, which develop slowly after a comparatively long latency (Nilsson et al., 1993), whereas mouse oligodendrocyte precursor cells exhibit rapid onset spikelike GABA responses (Kirchhoff and Kettenmann, 1992). GABA triggers responses mainly through the $GABA_A$ and partly through the $GABA_B$ receptor subtypes. The calcium rise results from internal store release and from influx through dihydropyridine-sensitive calcium channels opened by GABA-induced depolarization (Kirchhoff and Kettenmann, 1992; Nilsson et al., 1993).

Determinants of Response Pattern

Stimulus dose partly determines the shape of glial calcium responses. With increasing glutamate dose, response latency decreases and the amplitude of the initial spike increases (Ahmed et al., 1990). The frequency of evoked calcium oscillations increases until at high glutamate the predominant sustained response changes from an oscillatory pattern to a smooth plateau (Glaum et al., 1990; Cornell-Bell and Finkbeiner, 1991). Histamine responses are also dose-dependent: low histamine tends to evoke simple spikelike calcium transients, high histamine elicits a sustained component following the initial spike (Fukui et al., 1991). Increasing norepinephrine reduces the average latency calcium responses and shifts the predominant temporal pattern from a simple transient to a biphasic response (Salm and McCarthy, 1990). Low endothelin doses produce a transient calcium spike: at a slightly higher endothelin dose, a second sustained calcium rise appears briefly after termination of the initial spike. As the endothelin dose increases, the sustained component grows in amplitude and decreases in latency until it fuses with the initial spike to form a biphasic response (Goldman et al., 1991).

Receptor expression differences and receptor synergy also determine the shape of glial calcium responses. As an example of variable receptor expression, type-2 astrocytes respond to kainate, AMPA, and histamine more often than type-1 astrocytes (Fukui et al., 1991; Jensen and Chiu, 1991). However, type-1 astrocytes exhibit biphasic/polyphasic responses to glutamate, whereas type-2 astrocyte responses tend to be monophasic (Jensen and Chiu, 1991). In another example, α_1-adrenoreceptor density correlated with the fraction of rat cortical astrocytes showing phenylephrine calcium responses but did not correlate with response amplitudes (Shao and McCarthy, 1993). Interestingly, the receptor density varied widely among cultured astrocytes; even among astrocytes of the same lineage. Sometimes coapplication of two or more agonists produces calcium responses, which are more complex than the sum of the individual responses. For example, 2-chloroadenosine or the tachykinin receptor agonist, substance P, each elicits a transient calcium response (Delumeau et al., 1991). However, simultaneous substance P and 2-chloroadenosine application produces a nonadditive calcium rise that has a higher peak and significantly longer duration. Similarly, in mouse striatal astrocytes, somatostatin and methoxamine or 2-chloroadenosine stimulate transient calcium responses separately and a prolonged calcium response in combination (Marin et al., 1991b; El-Etr et al., 1992).

Acid/base status also plays a role in modulating ligand-induced calcium responses. Lowering extracellular pH substantially decreases ATP-evoked calcium influx in rat astrocytes (Neary et al., 1993). This may become important in certain pathological conditions which result in simultaneous tissue acidosis and ATP release (Neary and Norenberg, 1992). Certain stimuli, such as kainate and chemical depolarization, evoke simultaneous but independent changes in intracellular calcium and pH (Deitmer and Munsch, 1992; Deitmer et al., 1993).

SPATIAL ASPECTS OF GLIAL CALCIUM SIGNALING

Intracellular and Intercellular Calcium Waves

Fluorometric techniques reveal important spatial features to many glial calcium responses. Within a

single cell, calcium transients may appear in separate glial processes without involving the nucleus (S. Finkbeiner, unpublished observations). In other instances, calcium levels rise uniformly throughout the cytoplasm. Often glial calcium transients arise in one restricted part of a cell and propagate as a wave to other areas. The initial calcium spike of glutamate, quisqualate or histamine responses occurs first in the cell periphery and rapidly spreads to the nucleus within a second or two (Glaum et al., 1990; Cornell-Bell and Finkbeiner, 1991; Fukui et al., 1991). When viewed in sequence, the calcium rise appears to propagate from the edge of the cell to the center. This wave type occurs only once—during the initiation of a calcium response.

A second wave type may appear after the initial spike, during the sustained component of certain biphasic/polyphasic responses (Figure 17-4; Table 17-2). This intracellular calcium wave travels slowly with near-constant velocity and may recur multiple times. The initiation site and propagation path vary considerably from cell to cell, though recurrent waves frequently begin from the same location and propagate along the same path.

Intracellular calcium waves often cross cell boundaries, propagating through and between cells (Figure 17-5; Table 17-2). Intercellular calcium waves travel nearly 20 μm/s and may involve such large portions of a glial network that an exact measurement of the extent of some intercellular calcium waves is impossible. Glutamate-evoked intercellular waves depend on extracellular calcium: the incidence and spatial

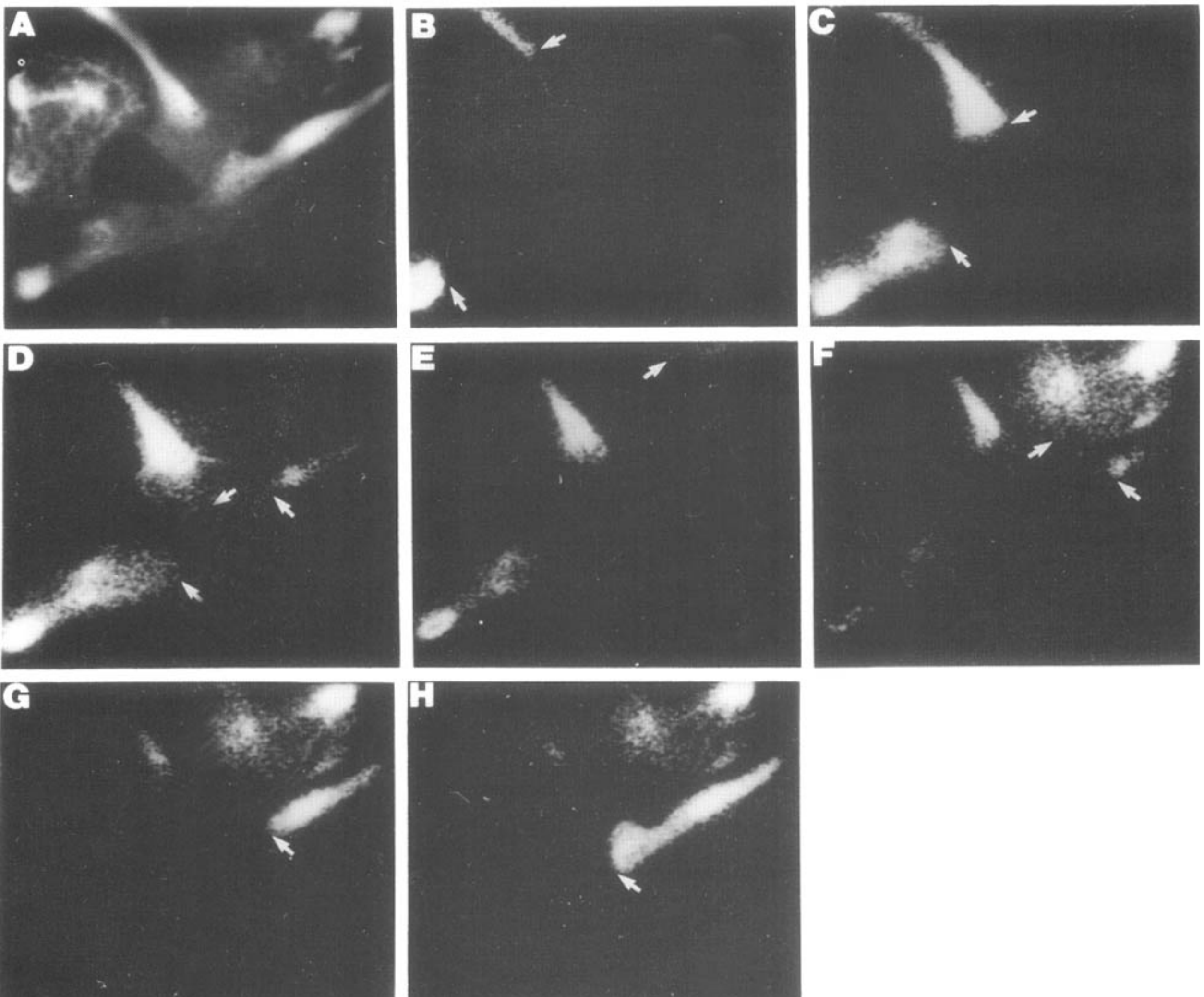

FIG. 17-4. Glial intracellular Ca^{2+} waves. (**A**) Fluorescence of four fluo-3-loaded rat hippocampal type-1 astrocytes prior to glutamate application. (**B–H**) Fluorescence-change images produced by digitally subtracting the baseline image (A) from images at 6-second intervals (6, 12, 18, 24, 30, 36, and 40 seconds) following glutamate application. *Arrows* indicate wave fronts. Scale bar = 100 μm. [Adapted from Cornell-Bell et al. (1990). Copyright (c) 1990 by AAAS. Reprinted by permission.]

TABLE 17-2. *Temporal and Spatial Aspects of Glial Ca^{2+} Signaling*

Stimulus	Oscillation Period(s)	Wave Velocity (μm/s) Intra-/Intercellular		Reference
AMPA	20–40	N/A	N/A	Cornell-Bell and Finkbeiner, 1991
ACPD	5–28	N/A	N/A	Cornell-Bell and Finkbeiner, 1991; Kriegler and Chiu, 1993
ATP	5–20	15	N/R	Kriegler and Chiu, 1993; van den Pol et al., 1992
Endothelin	10–90	N/A	N/A	Marin et al., 1991a
Glutamate	5–100	9–61	12–25	Charles et al., 1991, 1993; Cornell-Bell et al., 1990; Cornell-Bell and Finkbeiner, 1991; Fatatis and Russell, 1992; Finkbeiner, 1992; Jensen and Chiu, 1990, 1991; Kriegler and Chiu, 1993; van den Pol et al., 1992
Histamine	20–40	N/A	N/A	Fukui et al., 1991
Isoproterenol	25–50	N/A	N/A	Nilsson et al., 1991
Mechanical	7–45	28–42	15–27	Charles et al., 1991, 1993
Methoxamine	15–20	N/A	N/A	Marin et al., 1991b
Neuronal stimulation	16–30	7–27	8–10	Dani et al., 1992
Norepinephrine	20–80	N/A	N/A	Nilsson et al., 1991; Salm and McCarthy, 1990
Phenylephrine	8–24	N/A	N/A	Nilsson et al., 1991
Quisqualate	10–100	N/A	N/A	Cornell-Bell et al., 1990; Glaum et al., 1990; Jensen and Chiu, 1990
Serotonin	7–20	15	N/A	Deecher et al., 1993; Nilsson et al., 1991; van den Pol et al., 1992
Serum factors	18–45	N/A	N/A	van den Pol et al., 1992
Spontaneous	45–80	N/A	N/A	Kastritsis et al., 1992; Nilsson et al., 1991; Salm and McCarthy, 1990
Substance P	10–15	N/A	N/A	Martin et al., 1992

AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; ACPD, *trans*-1-aminocyclopentane-1,3-dicarboxylic acid; ATP, adenosine triphosphate; N/A, not applicable; N/R, not reported.

extent of intercellular waves dramatically drops in the absence of extracellular calcium (Cornell-Bell et al., 1990). However, mechanically stimulated glial intercellular waves do not depend on extracellular calcium (Charles et al., 1991).

Spatial Organization and Agonist Dose

Stimulus dose may partly determine the spatial organization of calcium responses. In hippocampal astrocytes, increasing glutamate appears to organize calcium responses over progressively larger spatial domains: circumscribed asynchronous calcium transients occur within single cells at low doses; intracellular and intercellular calcium waves occur at higher doses (Cornell-Bell et al., 1990; Cornell-Bell and Finkbeiner, 1991). Increasing histamine dose changes cortical astrocyte responses from asynchronous calcium increases limited to cell processes into transients which propagate along cell processes toward the nucleus (Fukui et al., 1991).

Oscillations, Waves, and Mechanistic Models

The biochemical mechanism which propagates calcium waves is unknown. Generally, propagation theories incorporate diffusion in two dimensions into existing calcium oscillation models to create calcium waves. This approach fits observations in oocytes and some other cell types well (Berridge, 1993), however, several results in glial cells are difficult to reconcile with existing models. There may be important differences between glutamate-evoked calcium oscillations and waves. Calcium oscillations

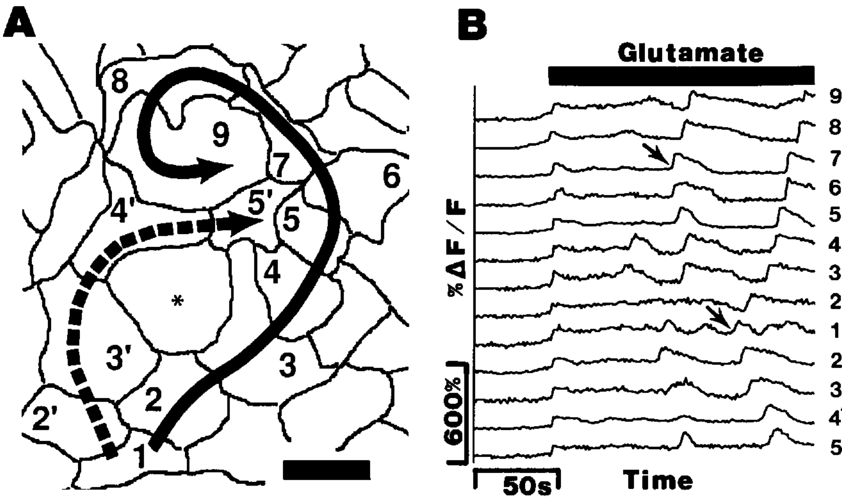

FIG. 17-5. Glial intercellular Ca^{2+} waves. (A) The outlined astrocyte labeled *1* initiates an intracellular Ca^{2+} wave, which propagates intercellularly (*arrow*). As the wave propagates into cells labeled *2* and *3'*, it encounters a natural barrier in the glial network, a glial cell that does not perpetuate the Ca^{2+} wave (*asterisk*). As the wave propagates around the nonparticipating cell, it effectively becomes two separate Ca^{2+} waves. Scale bar = 100 μm. (B) The Ca^{2+} responses of astrocytes from Figure *A* are depicted in the form of fluorescence-time course traces. Glutamate application (see addition bar at top of figure) first evokes a sharp Ca^{2+} transient, which appears as the initial spike in the trace of each astrocyte. In continued glutamate, some astrocytes initiate intracellular Ca^{2+} waves. Waves appear as abrupt rises in cytoplasmic Ca^{2+}, which peak early, plateau for 10 to 20 seconds, then return to baseline (e.g., *arrows*). Intercellular propagation appears as a regular succession of peaks in traces from adjoining astrocytes. For example, two intercellular Ca^{2+} waves are shown. The astrocyte labeled *7* initiates one intercellular Ca^{2+} wave (*first arrow*). The intercellular wave propagates in two directions: through cells labeled *8* and *9* and also through cells labeled *6* and *5* (the apparent involvement of cells labeled *3* and *4* is actually another Ca^{2+} wave which is initiated in cell *3* and propagates into cell *4*). As the wave propagates through the neighboring cells, the stereotypical cytoplasmic Ca^{2+} rise occurs with the peak phase shifted by a time interval that is proportional to the distance the wave traveled. The second intercellular wave (and the subject of Figure *A*) is initiated by the astrocyte labeled *1* (*second arrow*). [Adapted from Finkbeiner (1992), with permission.]

appear quickly after the initial spike, have a typical temporal pattern with a relatively slow rise and fall and an amplitude that is usually less than the initial spike. For a given cell, repeated stimulation often evokes nearly identical oscillatory responses and a wide variety of stimuli evoke similar, though not identical, oscillations. Early in an oscillatory response, calcium appears to rise and fall uniformly in all regions of a cell. As the oscillations continue, rises often become nonuniform: one subsection of the cell appears to lead the rest. Although the temporal profile of the calcium response maintains the original oscillatory shape, peaks of the oscillation sampled within the cell become phase-shifted, resulting in a wavelike appearance to the calcium rise. However, these "waves" propagate rapidly (30 to 60 μm/s); much quicker than intercellular calcium waves (15 to 20 μm/s).

By contrast, intercellular calcium waves are less common than oscillations and appear episodically rather than periodically following the initial spike. The shape differs from oscillations in that waves have an abrupt onset with a rapid rise to a peak amplitude similar to the initial spike. The duration is invariably longer than a typical oscillation such that the transient has a spike and plateau shape rather than the more sinusoidal shape of an oscillation. Recurrent calcium waves during a single stimulation are uncommon, and repeated stimulation rarely elicits recurrent waves.

Observations of apparent interactions between calcium oscillations and waves also do not fit well with current oscillation models. Occasionally, cells that propagate intercellular calcium waves will neighbor cells undergoing cytosolic calcium oscillations. In such cases, the oscillations often decrease in amplitude or frequency as the wave propagates nearby and may recur once the wave passes (Cornell-Bell and Finkbeiner, 1991). Curiously, adjoining astrocytes exhibiting calcium oscillations do not detectably coordinate responses and astrocytes, which organize to propagate mechanically stimulated calcium waves,

frequently show asynchronous calcium oscillations after the wave passes (Charles et al., 1991).

Although the details of calcium wave propagation are unclear, the data suggest several conclusions. The signal underlying wave propagation travels intracellularly. In support, calcium wave velocity and propagation direction are independent of the flow of superfusate, and calcium waves propagate around, rather than through, natural or artificial interruptions in the glial network (Enkvist and McCarthy, 1992; Finkbeiner, 1992). Short-range reaction-diffusion drives propagation rather than long-range diffusion, since the wave velocity remains constant over long distances (Finkbeiner, 1992). Depletion of IP_3-sensitive internal calcium stores with thapsigargin abolishes glutamate- or mechanically induced waves, suggesting IP_3-induced calcium release is necessary to permit propagation (Charles et al., 1993). Finally, the signal travels from cell to cell by gap junctions. Gap junction closure using cellular acidification, protein kinase C activation, or the gap junction blockers, octanol or halothane, sharply reduces intercellular propagation (Enkvist and McCarthy, 1992; Finkbeiner, 1992). In the case of octanol and halothane, calcium waves travel within cells, but are unable to travel between cells.

One important but unproven assumption holds that all calcium waves propagate by the same mechanism regardless of the initial stimulus. Although the assumption is probably valid, there are differences between glutamate-evoked and mechanically stimulated glial calcium waves. Most importantly, mechanically stimulated waves propagate unaffected in calcium-free media, whereas we have yet to observe a glutamate-evoked intercellular wave under the same conditions. Mechanically stimulated calcium waves also differ in that the wave generally spreads concentrically outward to involve most or all of the surrounding visible cells. Glutamate-induced waves generally involve fewer cells and may propagate along convoluted paths. In this regard, mechanically induced calcium waves are similar to waves evoked by kainate or sodium-free media (Finkbeiner, 1991). Mechanical stimulation may trigger the metabolism of other plasma membrane lipids besides phosphatidylinositol bisphosphate, such as sphingosylphosphorylcholine. Certain sphingosine metabolites trigger the release of both the IP_3-sensitive calcium pool as well as another guanosine triphosphate (GTP)-sensitive calcium pool in a permeabilized cell preparation (Gill et al., 1992). However, it is difficult to imagine how this difference could account for propagation differences many micrometers from the stimulation site.

What might account for the differences between calcium oscillations and calcium waves and the interactions between cells exhibiting the response types? One idea posits two IP_3-sensitive calcium pools, one immediately beneath the plasma membrane, the second deep within the cell (Berridge, 1992). Upon stimulation, IP_3 rises near the membrane and causes an efflux of calcium from the pool proximal to the membrane without releasing calcium from the deep pool. Instead, the empty proximal pool results in calcium release-activated channel activation and transmembrane calcium influx, which charges the deep and proximal pools (Penner et al., 1993). Oscillations begin when deep pools become calcium-loaded, sensitizing the IP_3 receptors so that ambient IP_3 levels are sufficient to trigger release. The negative and positive feedback of the calcium ion on the IP_3 receptor generates the oscillation (Amundson and Clapham, 1993). Calcium oscillations could occur uniformly throughout a cell or appear wavelike, as calcium release at one point diffuses to influence IP_3-gated calcium release nearby. However, the temporal pattern should not change fundamentally and should always resemble an oscillation. In this formulation, the deep store largely resides in the perinuclear area and tapers near the cell periphery. Therefore, the lack of synchrony among neighboring oscillators could result from impedance mismatch: the concentration of the biochemicals underlying the calcium cycle would not achieve sufficient concentrations near the periphery to diffuse through gap junctions and impose a rhythm on a neighboring cell. True calcium waves might occur when loaded calcium stores near the plasma membrane participate. They too may need to load calcium sufficiently to resensitize the IP_3 receptors to enable the pool to release calcium. Involvement of two pools could explain why the amplitude and duration of waves is greater than oscillations. A subplasma membrane pool would also abut gap junctions at sites of cell-cell contact and might be expected to facilitate intercellular wave propagation. Mechanical stimulation may be more effective at triggering the release of proximal as well as deep stores and thus evoke a wave without calcium loading. This model fails to explain how a wave propagating through one cell could inhibit the oscillations of a neighbor without directly causing a detectable calcium change in the oscillating cell.

Another proposal suggests that the transition between oscillation and wave occurs when intracellular calcium buffering transiently decreases. This could result from transient inhibition or reversal of the plasma membrane sodium-calcium exchanger, inhibition of calcium-ATPases on internal stores, occupation of cytoplasmic calcium binding sites or mod-

ulation of the number or capacity of internal calcium stores. The change would necessarily be transient, since calcium oscillations can precede and follow calcium waves. A decrease in calcium buffering would allow a given quantity of calcium flowing into the cytoplasm to achieve a higher concentration and exert more influence on calcium-sensitive structures. In effect, a sufficient decrease could enhance calcium diffusion and raise resting levels of cytoplasmic calcium. Oscillations would result when IP_3 release sites are functionally isolated from one another by calcium buffering: waves would arise when the internal stores are linked by enhanced two-dimensional calcium diffusion due to buffering capacity reduction. A buffering decrease would also increase the amplitude and duration of calcium waves compared with oscillations, as seen experimentally. It is difficult to explain mechanically stimulated waves with this model and it also fails to explain how calcium waves could inhibit calcium oscillations.

SECOND-MESSENGER CROSSTALK

Glial calcium changes influence a host of second-messenger systems and ion channels, which directly or indirectly feedback to modulate calcium signaling (see Chapter 34, this volume). The calcium ion is part of a web of second-messenger and ion channel crosstalk, which needs to be understood to predict the full response of glial cells *in vivo* (Figure 17-6).

A discussion of all calcium-regulated glial functions is beyond the scope of this chapter (for review see Finkbeiner, 1993). However, a few examples will serve to illustrate how crosstalk may importantly alter glial calcium responses. Receptor-stimulated inositol turnover often leads to protein kinase C activation in addition to cytoplasmic calcium increases. Protein kinase C can feedback to inhibit the receptor-event that led to its activation. Potent protein kinase C activators, phorbol esters, block calcium responses to norepinephrine or the muscarinic receptor agonist carbachol (Orellana et al., 1985; Pearce et al., 1988), quisqualate and glutamate (Jensen and Chiu, 1990; Finkbeiner, 1991), endothelin and the proteolytic enzyme thrombin (Tas and Koschel, 1990; Lin et al., 1992), and histamine (Fukui et al., 1991). In some cases, phorbol esters alter specific components of calcium responses. Phorbol esters block the initial calcium spike, but not the subsequent sustained response to quisqualate (Glaum et al., 1990) or to acetylcholine (Brooks et al., 1989). In support of a specific protein kinase C-mediated effect, inactive phorbol ester analogues do not alter calcium responses to norepinephrine (Pearce et al., 1988) or

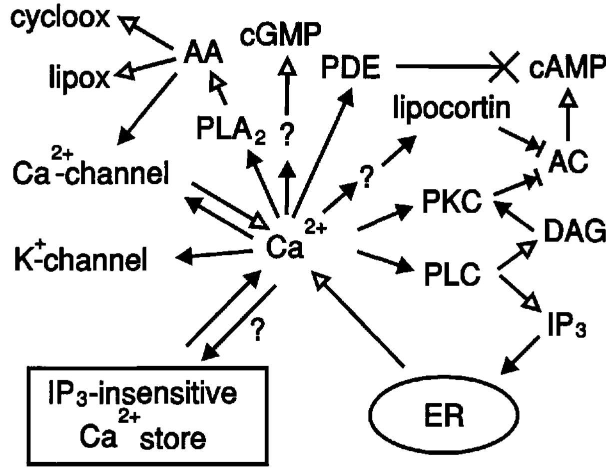

FIG. 17-6. Second-messenger crosstalk and glial Ca^{2+}, showing some of the known interactions between glial Ca^{2+} and other glial signaling systems. *Arrows with solid heads* show activation or production; *arrows with hollow heads* show release; *arrows with solid heads terminating in a perpendicular dash* indicate inhibition and arrows terminating in a cross show reduction. A *question mark* indicates uncertainty about the existence or basis of interaction. *ER*, endoplasmic reticulum; *IP_3*, inositol-1,4,5 trisphosphate; *PLC*, phospholipase C; *DAG*, diacylglycerol; *PKC*, protein kinase C; *AC*, adenyl cyclase; *cAMP*, cyclic adenosine monophosphate; *PDE*, phosphodiesterase; *cGMP*, cyclic guanine monophosphate; *AA*, arachidonic acid; *PLA_2*, phospholipase A_2; *cycloox*, cyclooxygenase; *lipox*, lipoxygenase.

thrombin (Tas and Koschel, 1990). The effect seems specific to stimuli that activate receptors coupled to phospholipase C, since phorbol esters do not inhibit spontaneous calcium spikes or agonist-induced calcium responses to kainate (Enkvist et al., 1989a; Jensen and Chiu, 1990).

Arachidonic acid- and calcium-mobilizing pathways interact in several ways. Glial calcium rises stimulate phospholipase A_2 to mobilize arachidonic acid and cyclooxygenase and lipoxygenase metabolites (Shirazi et al., 1989; Bruner and Murphy, 1990; Ishizaki and Murota, 1991). One way calcium rises may activate phospholipase A_2 is through protein kinase C-mediated phosphorylation of the endogenous phospholipase A_2 inhibitor, lipocortin (Murphy et al., 1985; Shirazi et al., 1989). Subsequently, arachidonic acid mobilization modulates glial calcium responses. Arachidonic acid evokes a slow cytoplasmic calcium rise in hippocampal, cortical, and striatal astrocytes (Delumeau et al., 1991; Finkbeiner, 1991) and activates a voltage-independent, nonselective calcium channel in rat oligodendrocytes (Soliven et al., 1993). Arachidonic acid may also modulate glial calcium indirectly. For example, arachidonic acid inhibits glial glutamate uptake, causing extracellular glutamate to accumulate sufficiently to activate glutamate receptors (Marin et al., 1991b; El-Etr et al., 1992; van den

Pol et al., 1992). *In vitro,* substance P or 2-chloroadenosine appears to potentiate methoxamine-evoked calcium responses by activating glutamate receptors through arachidonic-acid mediated inhibition of glutamate uptake (Marin et al., 1991b; El-Etr et al., 1992). Arachidonic acid also activates a glial potassium channel, which causes membrane hyperpolarization and thus enhances the electrochemical gradient for transmembrane calcium entry (Delumeau et al., 1991). These interactions may be particularly important *in vivo* where neurotransmitter and neuropeptide are often released at the same synapse (Cooper et al., 1991).

THE FUTURE: A CALCIUM-BASED GLIAL SIGNALING SYSTEM

Too little is known of calcium-regulated glial processes to guess how particular glial calcium response patterns might be translated into specific physiological responses. However, features of calcium responses, which glia exhibit singly and as a network, could form the basis for a calcium-based long-range signaling system *in vivo.* As components of the network, glial cells are capable of dependably encoding information such as the type and intensity of a stimulus. Glia are capable of encoding stimulus type through the combination of receptors that a particular cell expresses and through the percentage of cells that respond to a particular ligand (McCarthy and Salm, 1991; Nilsson et al., 1991; van den Pol et al., 1992; Deecher et al., 1993; Finkbeiner, 1993; Shao and McCarthy, 1993). Consequently, some stimuli produce monophasic responses, others biphasic responses, still others produce polyphasic responses with an almost infinite set of possible patterns. Glia can encode stimulus intensity through changes in the temporal pattern, such as oscillation frequency, or in the spatial extent of the response. Glia also show precisely reproducible responses (Finkbeiner, 1993). The property, called *fingerprinting,* refers to the fact that a given agonist tends to evoke reproducible calcium response patterns in a particular glial cell though the response patterns may differ widely between cells (Rooney et al., 1989). As a network, glia *in vitro* transmit calcium signals nondecrementally over long distances via propagating intercellular calcium waves. The network has a specific circuitry determined by the level of intercellular gap junction coupling (see Chapter 19, this volume). Glial networks also show plasticity, since glial connectivity can rapidly and reversibly respond to neuronally released substances which regulate gap junctional coupling (Enkvist and McCarthy, 1992). Other reviews discuss the plausibility of glial involvement in neural information processing (Smith, 1992; Finkbeiner, 1993).

Nearly all glial calcium response data comes from *in vitro* culture systems; however, recent discoveries suggest that glial calcium signaling occurs *in vivo.* *First,* glia often respond preferentially to neurotransmitters, neuropeptides, or other stimuli present in the particular brain region from which the cells are cultured (Somogyi et al., 1990; van den Pol et al., 1992). *Second,* glial cells *in situ* express and translate mRNA for a variety of ion channels and neurotransmitter receptors (Matute and Miledi, 1993). For example, Bergmann glia express glutamate receptors in processes adjacent to synapses, which release glutamate (Somogyi et al., 1990). *Third,* selective electrical stimulation of hippocampal neurons in organotypic slices elicits astrocytic calcium waves, presumably through local transmitter release (Dani et al., 1992). Organotypic slices are cultured brain slices, which retain some *in vivo* anatomical relationships. *Fourth,* acutely excised rat optic nerve astrocytes exhibit calcium spiking, which reflects the level of neuronal activity. *In situ* calcium responses to the exogenously applied glutamate analogues AMPA, ACPD, and kainate resembled responses obtained *in vitro* except no calcium waves were seen (Kriegler and Chiu, 1993).

CONCLUSIONS

In many ways glial control of intracellular calcium is similar to other cell types: energy-dependent pumps and exchangers maintain a steep calcium concentration gradient between the cytosol and the extracellular solution or internal calcium stores. Similar to other cells, stimuli activate calcium influx through several plasma membrane calcium channel types, release calcium from internal stores or do both. However, the sheer number of stimuli to which glia respond and the temporal and spatial complexity of calcium responses are unequaled. Although the significance of the particular patterns is unknown, response characteristics suggest a calcium-based glial signaling system, which is both robust and specific. Yet, many questions remain unanswered. Exactly what comprises glial calcium stores and how are they regulated? What, systems do glia possess to translate complex calcium response patterns into specific physiological responses? What is the neurobiological significance of astrocytic calcium wave propagation? Finally, what glial calcium signaling occurs *in vivo* and what is its purpose?

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18 Modulation of glial potassium, sodium, and chloride activities by the extracellular milieu

KLAUS BALLANYI

Activity in the central nervous system is accompanied by movements of ions and water across neuronal membranes that modify the composition of the adjacent extracellular space. The interposition of glial cells between the neurons, and the geometry and membrane properties of these cells, have led to the suggestion that an important role of glial cells is to contribute to the homeostasis of the extracellular fluid (Orkand et al., 1986; Coles, 1989; Walz, 1989). It is well known that enhanced neuronal activity is accompanied by transient elevations of extracellular K^+ activity (aK_e) (Somjen, 1979; Chapter 26, this volume). Such alterations of aK_e can perturb neuronal functions (Sykova, 1983). Measurements of glial membrane potentials, aK_e, and extracellular space volume during physiological or electrical stimulation of neurons have established the idea that glial cells are responsible for K^+ buffering in the brain. Quantitative aspects of these relations between neuronal and glial responses are given in Chapters 11, 26, 31, 47, and 62 (see also Dietzel et al., 1989).

Until intracellular ion-sensitive microelectrodes were used, it was not clear to what extent K^+ uptake might affect the intracellular ion milieu of glia. Double-barreled, ion-sensitive microelectrodes monitor membrane potentials and, simultaneously, cellular ion activities (Ammann, 1986). This gives information about relevant physiological processes since the transmembraneous gradients of ion activities, rather than those of total ion concentrations, determine equilibrium potentials for different ions and, therefore, membrane potentials and cellular functions.

This chapter concentrates on the intracellular activities of K^+, Na^+, and Cl^- (aK_i, aNa_i, aCl_i), since the homeostasis of these ions is essential for a variety of glial properties, including membrane potential, cell volume, and K^+ buffering. Most of the data on modulation of glial aK_i, aNa_i, and aCl_i stem from *in vitro* studies on (1) astrocytes in brain slices, (2) cultured astrocytes or oligodendrocytes, (3) glial cells of the medicinal leech, and (4) pigment epithelial glial cells in the bee retina (see Table 18-1 for references).

RELATION BETWEEN INTRACELLULAR ION ACTIVITIES AND ION CONTENT

A quantitative comparison of glial ion activities and ion content in the bee retina indicates that intracellular K^+, Na^+, and Cl^- are freely dissolved in the cytosol (Coles et al., 1986; Orkand et al., 1986). A similar situation is believed to exist in glial cells of other nervous tissues. Accordingly, recordings with microelectrodes, selective for these ions, can also be labeled in free concentrations, by dividing activities by an activity coefficient (γ) of approximately 0.7 (Ammann, 1986; Orkand et al., 1986). Changes in ion activities are due only to transmembrane fluxes of ions or water, not to exchange with binding sites. This is in contrast to intracellular Ca^{2+} or H^+, which are predominantly bound or sequestered (Coles, 1989; Chapter 17, this volume).

LIMITATIONS OF MEASUREMENTS OF INTRACELLULAR ION ACTIVITIES

The quantitative determination of intracellular ion activities can, for example, suffer from junction potentials on the reference barrel of the ion-sensitive microelectrodes (Orkand et al., 1986; Coles et al., 1989) or from interference of other cellular ions with the ion-sensitive ligands (Ammann, 1986). Using the classical Corning ion-exchanger as a K^+ sensor, measurements of aK_i can be distorted by a high aNa_i or by intracellular substances with a quaternary ammonium-based structure like acetylcholine or serotonin (Ammann, 1986). In glial cells of the medicinal leech (Schlue and Wuttke, 1983) or of the bee retina (Coles and Orkand, 1983), resting aK_i

TABLE 18-1. *Steady-State Values of Membrane Potentials and the Calculated Equilibrium Potentials for the Intracellular Activities of Potassium, Sodium, and Chloride Ions**

Parameter	Astrocytes Guinea Pig Olfactory Cortex	Astrocytes Mouse (Culture)	Oligodendrocytes Mouse (Culture)	Unidentified Glia Frog Spinal Cord	Epithelial Glia Drone Bee Retina	Neuropile Glia Leech Segment Ganglium
E_m (mV)	−85[a]	−74[b]	−70[d]	−65[g]	−55[h]	−69[k]
aK_i (mM)	66[a]	75[b]	74[d]	75[g]	56[h]	70[k]
E_K (mV)	−87	−86	−70	−75	−81	−80
aNa_i (mM)	25[a]		15[e]	23[g]	37[i]	10[k]
E_{Na} (mV)	+37		+52	+35	+38	+60
aCl_i (mM)	6[a]	20 to 40[c]	9[f]	8[g]	13[j]	7[k]
E_{Cl} (mV)	−86	−48 to 30	−60	−65	−65	−67

*As measured with double-barreled, ion-sensitive microelectrodes in glial cells of different nervous tissues. Data were taken from the following references.
[a]Ballanyi et al., 1987.
[b]Walz, 1992.
[c]Kettenmann, 1987.
[d]Kettenmann et al., 1983.
[e]Ballanyi and Kettenmann, 1990.
[f]Hoppe and Kettenmann, 1989a.
[g]Bührle and Sonnhof, 1983.
[h]Coles and Tsacopoulos, 1979.
[i]Coles and Orkand, 1985.
[j]Coles and Schneider-Picard, 1989.
[k]Ballanyi and Schlue, 1989.

appears to be up to 20% higher when measured with the Corning exchanger instead of a neutral carrier, which is highly selective for K^+. Even greater discrepancies have been reported for resting levels of aK_i in glial cells of the frog spinal cord (Bührle and Sonnhof, 1983). In astrocytes of guinea pig olfactory cortex (Ballanyi et al., 1987), aK_i was monitored with Corning microelectrodes, which, due to the small size of their tips, had only a negligible sensitivity to quaternary ammonium ions (Ammann, 1986). A precise determination of glial aCl_i is hindered by interferences from intracellular anions like HCO_3^-. In low Cl^- solutions, such anions produce an electrode signal corresponding to an apparent aCl_i of 0.5–4 mM (Ballanyi et al., 1987; Kettenmann, 1987; Coles et al., 1989; Hoppe and Kettenmann, 1989a; Ballanyi and Schlue, 1990) (Figures 18-2 and 18-3). In contrast, interference of aNa_i measurements from cellular cations like Ca^{2+} is assumed to be rather small (Coles and Orkand, 1985; Ballanyi et al., 1987). Intraglial Na^+ in Na^+-free solutions can decrease to an apparent aNa_i of less than 1 mM in cultured mouse oligodendrocytes (Ballanyi and Kettenmann, 1990).

RESTING LEVELS OF aK_i AND aNa_i

Despite differences in the composition and ionic strength of the superfusion media, the K^+ and Na^+ gradients and, therefore, the equilibrium potentials for K^+ and Na^+ (E_K, E_{Na}), as derived from the Nernst equation, are similar in glial cells of different preparations (Table 18-1). Glial membrane potential (E_m) is close to E_K (Figure 18-2) which is in line with the assumption that, under resting conditions, K^+ channels in these cells contribute to a much higher degree to membrane conductance than Na^+ and/or Ca^{2+} channels (Orkand et al., 1966). It appears that aK_i is lower and aNa_i is higher in glial cells than in neurons, as measured in preparations that allow recordings from both types of brain cells (Coles and Tsacopoulos, 1979; Coles and Orkand, 1983, 1985; Ballanyi et al., 1987; Grafe and Ballanyi, 1987; Ballanyi and Schlue, 1990; Dörner et al., 1990) (Figure 18-1).

RESTING LEVEL OF aCl_i

In several types of glial cells *in situ*, aCl_i indicates a passive distribution of Cl^- (Bührle and Sonnhof, 1983; Ballanyi et al., 1987; Ballanyi and Schlue, 1990; Wuttke, 1990). This means that E_{Cl} is almost identical to E_m and aCl_i can rapidly follow changes of glial E_m (Figures 18-2 and 18-3). Such passive Cl^- movements require a significant Cl^- conductance as has been reported for leech neuropile glial cells (Schlue and Walz, 1984) and for a subpopulation of cultured oligodendrocytes of mouse spinal cord (Kettenmann, 1987). Inwardly directed Cl^- pumps seem to provide an aCl_i in retinal glial cells (Coles et al., 1989) and in a major population of cultured mouse oligodendrocytes (Hoppe and Kettenmann,

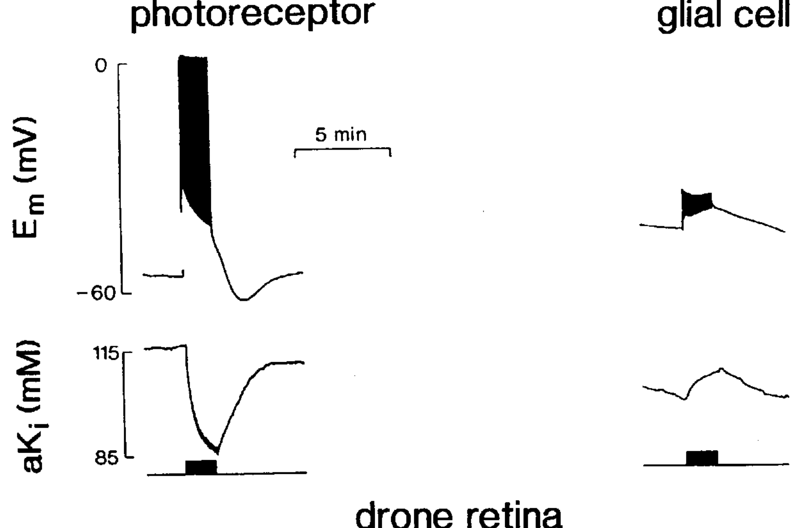


FIG. 18-1. Light-evoked electrical responses and changes in intracellular K^+ activity (aK_i) in the drone retina. The top trace is the membrane potential (E_m) of a photoreceptor (*left traces*) and a glial cell (*right traces*) recorded by the reference barrel of a double-barreled microelectrode. The *middle trace* is the difference in potential between the top trace and that of the barrel containing the K^+ sensor and gives aK_i. The *solid bars* on the lowest trace indicate when the retina was stimulated with 20-ms flashes of light at 1 Hz for 90 seconds. Note that aK_i decreases in the photoreceptor and increases in the glial cell, although both types of brain cells are depolarized in response to such stimulation. [From Orkand et al. (1986), with permission.]

1989a, 1989b), which is 2 to 5 mM higher than expected from a passive Cl^- distribution (Kettenmann, 1987) (Figure 18-3). In contrast to these reports on a rather low intracellular Cl^-, aCl_i is up to 40 mM above electrochemical equilibrium in cultured mouse astrocytes, indicating the activity of an inwardly directed Cl^- pump (Kettenmann, 1987) (Figure 18-3). In summary, the data on aCl_i suggests that Cl^- pumps might be expressed in a variety of glial cells. Their activity, however, is possibly masked to a more or less degree by short-circuiting Cl^- conductances, as has been demonstrated for mammalian skeletal muscle (Aickin et al., 1989).

K^+-DEPENDENT MODULATION OF aK_i, aNa_i, AND aCl_i

Excitatory synaptic activity results in an opening of transmitter- and voltage-gated neuronal ion channels. This evokes a Na^+ influx and a K^+ efflux, which can lead to a rise of neuronal aNa_i and a concomitant fall of aK_i (Orkand et al., 1986; Grafe and Ballanyi, 1987; Dörner et al., 1990) (Figure 18-1). As a result of such a neuronal K^+ release, aK_e can increase by several mM (Somjen, 1979; Sykova, 1983; Chapter 26, this volume). This induces a depolarization of the adjacent glial cells which is due

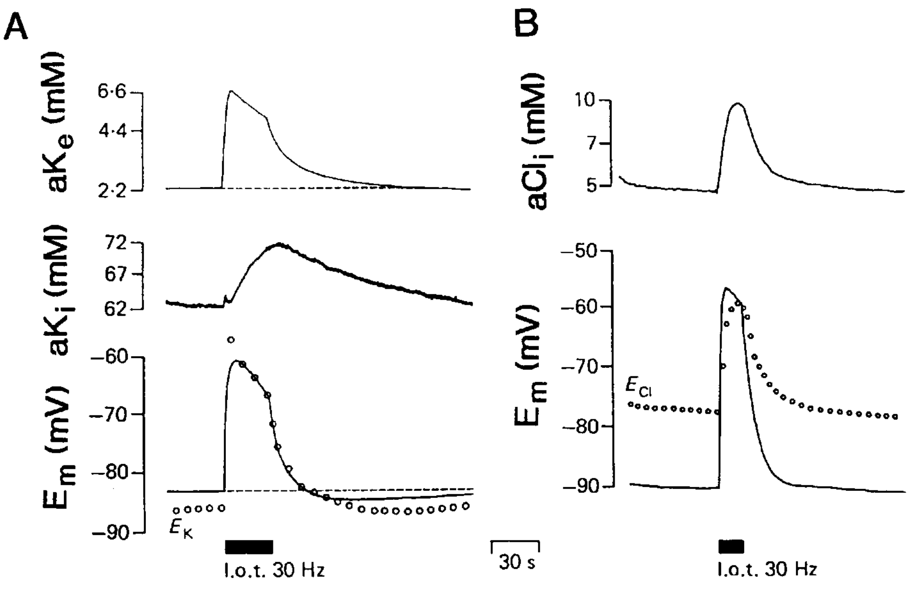
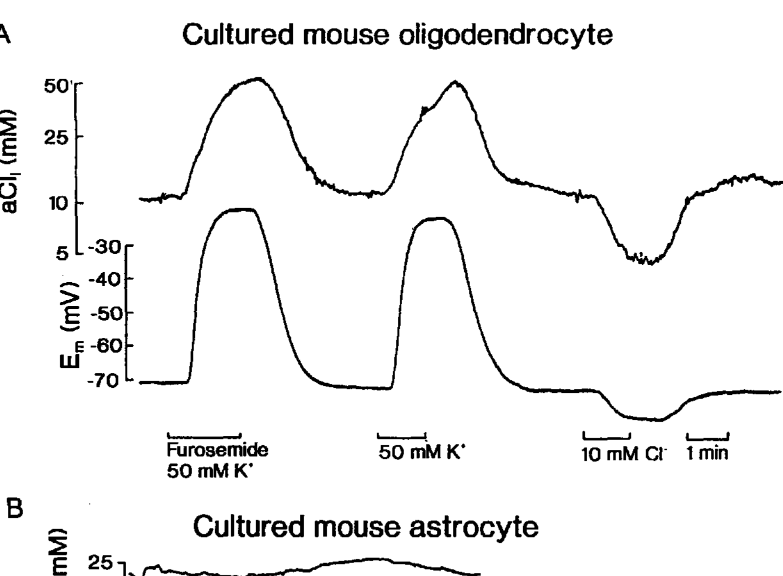


FIG. 18-2. (**A, B**) Electrical stimulation of the lateral olfactory tract (*l.o.t.*) of guinea pig olfactory cortex slices leads to increases of the intracellular activities of K^+ (aK_i) and Cl^- (aCl_i) in astrocytes. During the depolarization, the equilibrium potentials of K^+ (E_K) and Cl^- (E_{Cl}), as calculated using the measured values of aK_i and aCl_i, are almost identical to the membrane potential (E_m). The discrepancy between E_m and E_K before and after the stimulation indicates a contribution of Na^+ and/or Ca^{2+} conductances to resting E_m. The apparent discrepancy between E_{Cl} and E_m under resting conditions is due to interference of the Cl^- sensor from unknown intracellular anions. [From Ballanyi et al. (1987), with permission.]



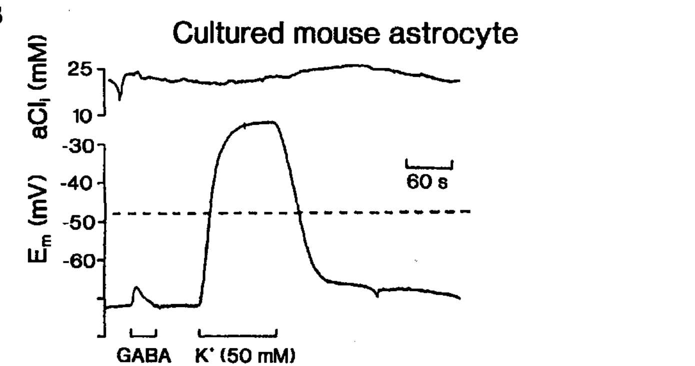


FIG. 18-3. (A) Transmembrane Cl^- movements in an oligodendrocyte, induced by changes in the extracellular concentration of K^+ ($[K^+]_0$) or Cl^- ($[Cl^-]_0$). *Top trace* displays intracellular Cl^- activity (aCl_i); *bottom trace*, membrane potential (E_m). Furosemide (10^{-4} M) in elevated $[K^+]_0$ and low $[Cl^-]_0$ (10 mM) were applied by exchange of the bathing fluid. The apparent hyperpolarization of E_m in low $[Cl^-]_0$ was caused by interference of this solution with the reference electrode. (B) aCl_i of an astrocyte. GABA (1 mM) and elevated $[K^+]_0$ were applied as indicated by the bars. The Cl^- equilibrium potential was calculated based on the measurement of aCl_i at the beginning of the trace and is indicated by the broken line. Note that the K^+-evoked depolarization leads to massive increases of aCl_i in the oligodendrocyte, but not in the astrocyte. [From Kettenmann (1987), with permission.]

to their high K^+ conductance (Orkand et al., 1966; Chapters 11 and 47, this volume). The K^+-induced glial depolarizations are accompanied by an increase of aK_i (Figures 18-1 and 18-2). Such glial K^+ uptake was originally demonstrated for Müller glial cells in the frog retina (Mori et al., 1976) and has been confirmed for glial cells in a variety of other nervous tissues (Coles and Tsacopoulos, 1979; Kettenmann et al., 1983; Schlue and Wuttke, 1983; Ballanyi et al., 1987; Walz, 1992). During these glial depolarizations, E_m is almost identical to E_K, which can precisely be determined when aK_e and aK_i are measured simultaneously (Figure 18-2) (see also Kettenmann et al., 1983; Kettenmann, 1987). The K^+-dependent increases of aK_i are mirrored by decreases of aNa_i as originally observed in the bee retina (Coles and Orkand, 1985) and later also in glial cells of mammals (Ballanyi et al., 1987; Ballanyi and Kettenmann, 1990) and of the medicinal leech (Ballanyi and Schlue, 1989). In the majority of glial cells investigated, the K^+-evoked changes of aK_i and aNa_i are accompanied by an increase of glial aCl_i (Ballanyi et al., 1987; Kettenmann, 1987; Ballanyi and Schlue, 1989, 1990; Coles et al., 1989; Hoppe and Kettenmann, 1989a; Wuttke, 1990) (Figures 18-2 and 18-3). The magnitude of the K^+-related changes of aK_i, aNa_i and aCl_i depends on the amplitude and duration of the activity-induced rises of aK_e and, therefore, glial depolarizations.

MECHANISMS OF K^+-EVOKED CHANGES OF aK_i, aNa_i, AND aCl_i

Donnan-like Influx of Potassium Chloride

A major portion of glial K^+ uptake appears to occur via K^+ and Cl^- influx through (Ba^{2+}-sensitive) K^+ and through Cl^- channels (Orkand et al., 1986; Ballanyi et al., 1987; Kettenmann, 1987; Walz, 1989). These transmembrane KCl fluxes can be explained by a passive Donnan-like redistribution of K^+ and Cl^-. During the initial phase of extracellular K^+ elevation, K^+ enters the glial cells when E_K is more positive than E_m, which is "clamped" to a more negative value by the Cl^- conductance (see also Chapter 26). Cl^- then enters the cells to establish a passive Cl^- distribution (Ballanyi et al., 1987; Kettenmann, 1987; Coles et al., 1989). In glial cells which are assumed to have a rather low Cl^- conductance at resting E_m, it was proposed that activation of voltage-dependent Cl^- conductances, as originally found in cultured rat astrocytes (Bevan et al., 1985), might mediate such passive Cl^- influx at depolarized potentials

(Kettenmann, 1987; Coles et al., 1989; Walz, 1989). However, even K^+-evoked depolarizations of up to 50 mV amplitude had almost no effect on aCl_i in cultured mouse astrocytes (Kettenmann, 1987; cf. Chapter 13).

Glial Swelling Evoked by Passive Potassium Chloride Influx

For osmotic balance, a passive accumulation of KCl, as described above, needs to be accompanied by an equimolar influx of water into the glial cells. This should lead to cell swelling (Orkand et al., 1986; Ballanyi and Grafe, 1988). Electrophysiological measurements of volume changes in leech neuropile glial cells have indeed shown that K^+-evoked swelling and shrinkage can be explained by a Donnan-like redistribution of KCl and water (Ballanyi and Grafe, 1988; Ballanyi et al., 1990; Wuttke, 1990) (Figure 18-4). Similar results were recently reported for cultured mouse astrocytes (Walz, 1992). However, if a passive KCl and water uptake were the only mechanism for glial K^+ accumulation, only a small increase of aK_i would be expected, since the amount of KCl which is passively taken up by the glia is isotonic. If K^+ was the only intracellular cation, such K^+ accumulation would not affect aK_i at all as the K^+ influx would completely be compensated by the accompanying influx of water (for details, see Orkand et al., 1986; Ballanyi and Grafe, 1988). Such glial swelling has a diluting effect on aNa_i which is assumed to be responsible for a major portion of the K^+-induced decrease of aNa_i (Wuttke, 1990). However, in various types of glial cells, the K^+-evoked decrease of aNa_i is bigger than that expected from a dilution as induced by cell swelling (Ballanyi and Kettenmann, 1990) (Figure 18-5). These considerations suggest that a different mechanism, involving an outward transport of Na^+, coupled to an inward transport of K^+, is involved in K^+-evoked glial K^+ uptake.

Sodium/Potassium Pump

The glial Na^+/K^+ pump is activated by excess of extracellular K^+ (Walz, 1989; Chapter 16, this volume). This becomes evident, when channel-mediated KCl uptake is inhibited by Ba^{2+}, which blocks glial K^+ conductances (Ballanyi et al., 1987; Ballanyi and Schlue, 1990). In the presence of Ba^{2+}, the remaining K^+-induced increases of aK_i (and the corresponding decreases of aNa_i) are blocked after addition of ouabain, which is an inhibitor of the Na^+/K^+ pump (Ballanyi et al., 1987; Grafe and Ballanyi, 1987). Similarly, ouabain blocked a slowly developing component of the K^+-evoked aNa_i decrease in cultured oligodendrocytes (Ballanyi and Kettenmann, 1990) (Figure 18-5).

It is assumed that during each pump-cycle, more Na^+ than K^+ is transported across the membrane (Ballanyi et al., 1987; Kettenmann, 1987; Walz, 1989; Chapter 16, this volume). Therefore, the electrogenic Na^+/K^+ pump tends to hyperpolarize the glial cells. For electroneutrality and osmotic balance, the net efflux of Na^+ needs to be compensated by a passive efflux of Cl^- and water. Accordingly, aCl_i has been demonstrated to decrease during Na^+/K^+ pump-evoked hyperpolarizations in astrocytes (Ballanyi et al., 1987). The Na^+/K^+ pump, therefore, contributes to the regulation of cell volume (Ballanyi and Grafe, 1988).

Na^+-K^+-$2Cl^-$ Cotransport

Measurements of ion fluxes with radiotracer methods have suggested that a substantial portion of K^+-related glial K^+ and Cl^- accumulation might be mediated by a Na^+-K^+-$2Cl^-$ cotransport (Walz, 1987, 1989, Chapter 13, this volume). However, a contribution of such K^+ transport to K^+-induced glial Cl^- uptake is not evident from measurements with ion-sensitive microelectrodes, since the increases of aCl_i are not affected by selective transport inhibitors such as furosemide and bumetanide (Kettenmann, 1987; Ballanyi and Schlue, 1990) (Figure 18-3). These inhibitors, however, blocked the recovery of aCl_i after exposure to solutions with a low Cl^- concentration. This indicates that Na^+-K^+-$2Cl^-$ cotransport is involved in the processes contributing to the maintenance of resting aCl_i (Hoppe and Kettenmann, 1989a). A detailed view of the function of anion transporters (and channels) in glia is given in Chapter 13.

Spatial Buffering

In addition to the K^+ uptake mechanisms described above, "spatial buffer" currents contribute to K^+ redistribution in the central nervous system in particular when activity-related increases of extracellular K^+ around the glia are not uniform (Orkand et al., 1966). This glial K^+ transfer might also affect aK_i, aNa_i, or aCl_i (Orkand et al., 1986). Spatial buffering is particularly relevant in certain central nervous system regions like the retina as discussed, for example, in Chapters 11, 31, 47, and 62.

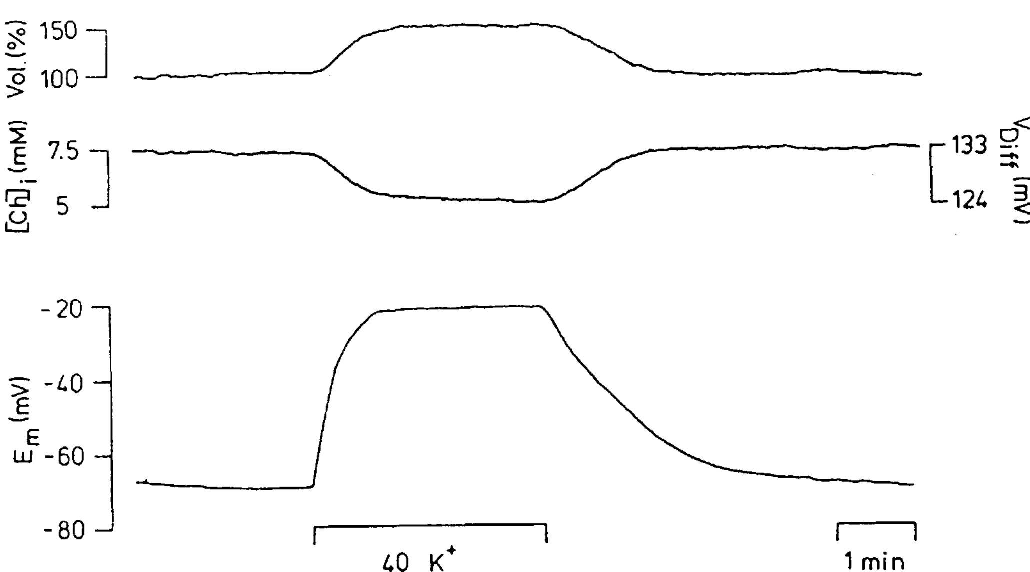


FIG. 18-4. Relationship between a K^+-induced change in the intracellular free choline (Ch) concentration ($[Ch]_i$), relative cell volume (Vol.) and membrane potential (E_m) in a leech neuropile glial cell. The cell was loaded with Ch via bath application (5 mM, 1 minute) about 5 minutes prior to the recording. Elevation of the extracellular K^+ concentration from 4 to 40 mM led to a prominent depolarization and a concomitant decrease of $[Ch]_i$. This reversible decrease corresponds to a cell swelling of 50% (for details, see Ballanyi and Grafe, 1988). The volume trace is identical with the $[Ch]_i$ trace (opposite polarity). V_{Diff} is the difference signal of the double-barreled ion-sensitive microelectrode, filled with the Corning K^+-exchanger. [From Ballanyi et al. (1990), with permission.]

K^+-Independent Modulation of aK_i, aNa_i, and aCl_i

In addition to the effects of changes in extracellular K^+, glial depolarizations or hyperpolarizations can be elicited by the direct action of neurotransmitters, extracellular ions like HCO_3^- or by drugs, affecting glial membrane conductances and/or ion transporters (see Chapters 14 and 21). During Ba^{2+}-induced depolarizations, aK_i and aCl_i can increase by more than 10 mM with a concomitant and sustained decrease of aNa_i (Figure 18-6) (see also Ballanyi et al., 1987). The increase of aK_i results from a Ba^{2+}-evoked blockade of K^+ channels (see above), which leads to a reduction of outward leakage of K^+. Ongoing Na^+/K^+ pump activity then produces accumulation of intracellular K^+. The fall in aNa_i can be explained by a reduced driving force for inward leakage of Na^+ in combination with Na^+ extrusion via the Na^+/K^+ pump. The increase of aCl_i is due to a passive redistribution of Cl^-.

Depolarizations of mouse oligodendrocytes, evoked by intracellular injection of Na^+ or Li^+, lead to profound decreases of aK_i as a result of an increased driving force for K^+ outward movements via K^+ channels (Kettenmann et al., 1987). A similar decrease of aK_i by up to 30 mM, and corresponding increases of aNa_i and aCl_i were revealed during glial depolarizations, evoked by activation of glial non-NMDA glutamate (Ballanyi and Kettenmann, 1990; Dörner et al., 1990) or nicotinic acetylcholine receptors (Ballanyi and Schlue, 1989), which are presumably coupled to nonspecific cation conductances (see Chapter 21, this volume). In a major population of cultured mouse oligodendrocytes, GABA activates Cl^- channels, which leads to a depolarization and to a concomitant decrease of aCl_i (Hoppe and Kettenmann, 1989b).

Glial hyperpolarizations can, for example, be elicited by serotonin (Schlue and Walz, 1984). The serotonin-induced hyperpolarization is accompanied by a passive fall of aCl_i (Ballanyi and Schlue, 1990) and a glial shrinkage of more than 10% (Ballanyi et al., 1990), whereas aK_i and aNa_i remain almost unaffected (Figure 18-7) (see also Brune et al., 1992).

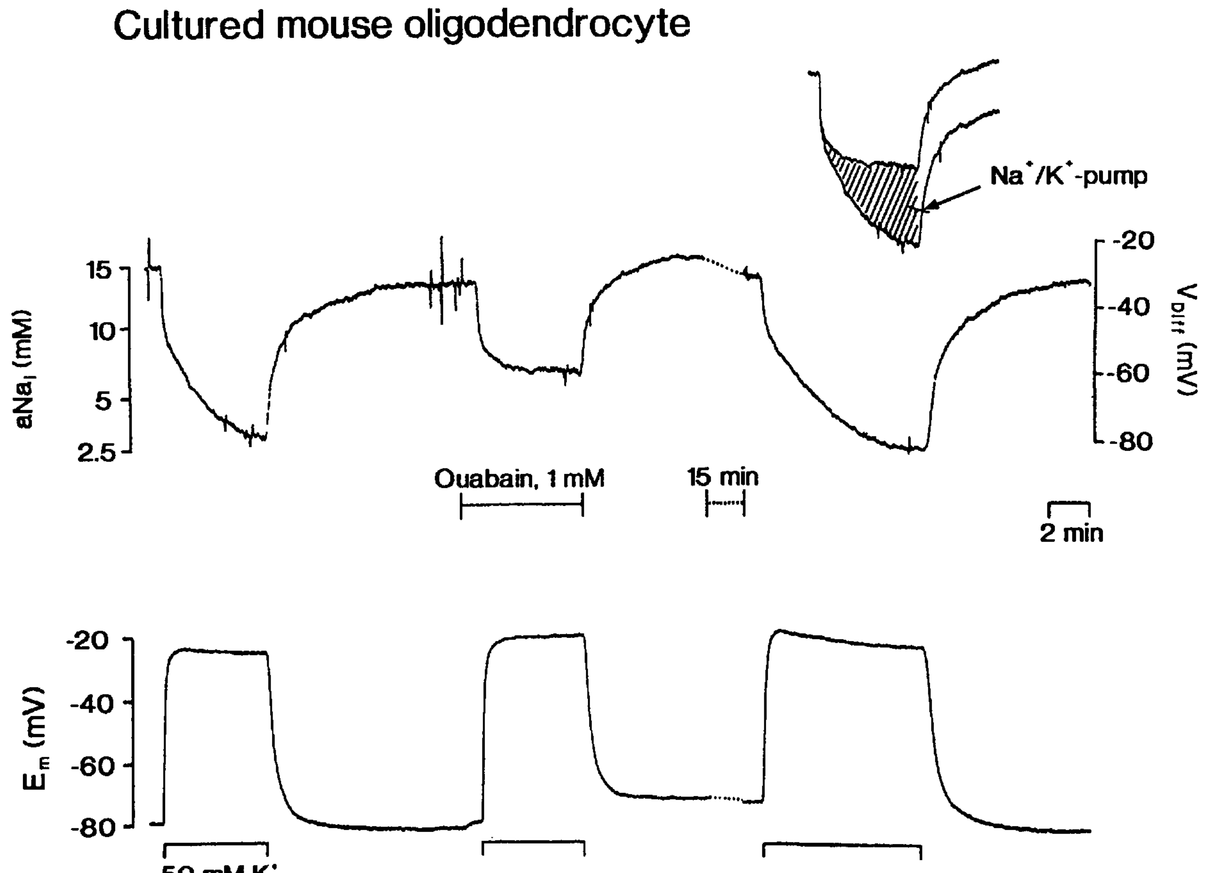


FIG. 18-5. Ouabain blocks the slow component of the K^+-induced decrease of intracellular Na^+ activity (aNa_i) in a cultured mouse oligodendrocyte. In this cell, ouabain had only a small effect on resting aNa_i and membrane potential (E_m). The *inset* shows superimposed traces of the aNa_i decrease in the control situation and in the presence of ouabain. The marked area indicates the contribution of the Na^+/K^+ pump to the K^+-induced fall of aNa_i. [From Ballanyi and Kettenmann (1990), with permission.]

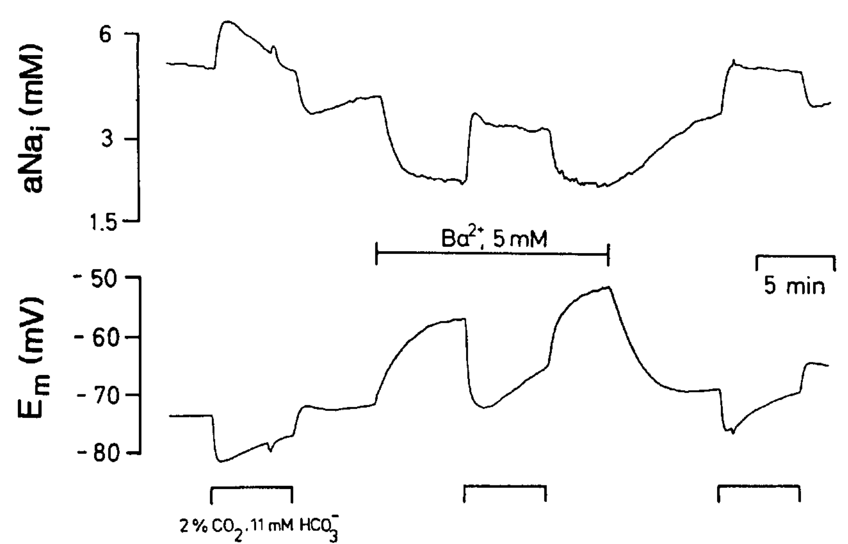


FIG. 18-6. Changing the superfusion medium from a CO_2/HCO_3^--free, HEPES-buffered solution to a saline, buffered with 2% CO_2/11 mM HCO_3^-, produced a hyperpolarization and an increase of intracellular Na^+ activity (aNa_i) in a leech neuropile glial cell. Both these effects are due to activation of an inwardly directed, electrogenic Na^+-HCO_3^- cotransport (see Deitmer and Schlue, 1989). Subsequent to the recovery, addition of Ba^{2+} produced a depolarization of membrane potential (E_m) by blockade of K^+ channels. The concomitant decrease of aNa_i can be explained by a reduced inward leakage of Na^+. In the presence of Ba^{2+}, both the CO_2/HCO_3^--induced hyperpolarization and aNa_i increase are potentiated in amplitude. [K. Ballanyi, unpublished observations.]

The hyperpolarization is due to activation of a serotonin receptor-coupled K^+ conductance, leading to a K^+ efflux. This K^+ efflux is paralleled by an equimolar efflux of Cl^- and of water which explains the shrinkage of the cells. aK_i does not change, since the loss of K^+, relative to the total content of dissolved cell K^+, is similar to the percentage change of cell volume (see above). aCl_i decreases despite the concentrating effect of the cell shrinkage, as it is much lower than aK_i (for details, see Orkand et al., 1986; Ballanyi and Grafe, 1988).

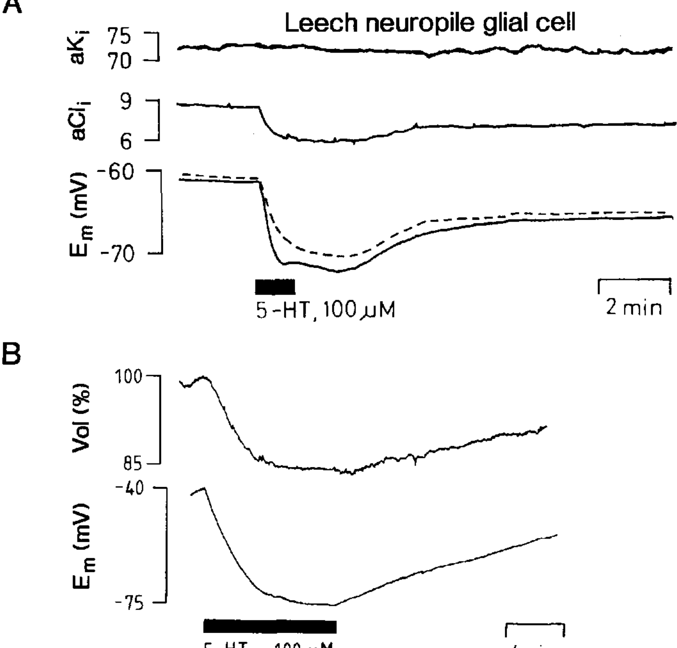
A similar passive decrease of aCl_i can be observed during hyperpolarizations of leech neuropile glial cells, elicited by changing the superfusion medium of the ganglia from salines which are nominally CO_2/HCO_3^--free to solutions containing CO_2/HCO_3^- (Ballanyi and Schlue, 1990). However, in contrast to the serotonin-induced hyperpolarizations, CO_2/HCO_3^--dependent hyperpolarizations lead to an increase of aNa_i and aK_i in these cells (Deitmer and Schlue, 1989; Brune et al., 1992) (Figure 18-6). The CO_2/HCO_3^--related hyperpolarization and aNa_i increase can be explained by activation of an inwardly directed, electrogenic Na^+-HCO_3^- cotransport (Deitmer and Schlue, 1989; Chapter 14, this volume), whereas the mechanism of the concomitant increase of aK_i remains to be analyzed (Brune et al., 1992).

Finally, it should be mentioned that glial ion activities can be modulated without an apparent change of glial E_m. In pigment epithelial glia of the drone retina, long-term illumination at physiological intensities evokes a maintained increase of aK_i subsequent to the recovery of aK_e and E_m from their initial and transient light-evoked perturbances (Coles and Schneider-Picard, 1989). The authors speculated that some unidentified physiological transmitters might have electrically silent effects on the glia (see also Chapter 52).

CONCLUSIONS

In glial cells of different central nervous system preparations, the activities of K^+, Na^+, and Cl^- (aK_i, aNa_i, aCl_i) undergo rather substantial changes during changes of the extracellular milieu (Figure 18-8). K^+, released by the neurons, elicits significant increases of glial aK_i and aCl_i as well as decreases of aNa_i, confirming the relevance of glial K^+ buffering for ion homeostasis of the brain. Neurotransmitters, as well as metabolites, that are also released into the extracellular space during physiological or pathophysiological processes in the brain, can directly evoke sustained changes of glial aK_i, aNa_i, and aCl_i independently and, in several cases, opposite to those during elevation of extracellular K^+.

I would like to thank Drs. D. W. Richter, G. Burckhardt, and S. W. Schwarzacher for a critical reading of the manuscript.



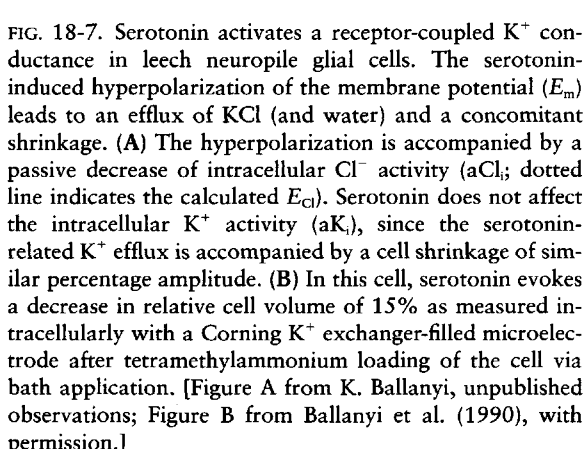


FIG. 18-7. Serotonin activates a receptor-coupled K^+ conductance in leech neuropile glial cells. The serotonin-induced hyperpolarization of the membrane potential (E_m) leads to an efflux of KCl (and water) and a concomitant shrinkage. (**A**) The hyperpolarization is accompanied by a passive decrease of intracellular Cl^- activity (aCl_i; dotted line indicates the calculated E_{Cl}). Serotonin does not affect the intracellular K^+ activity (aK_i), since the serotonin-related K^+ efflux is accompanied by a cell shrinkage of similar percentage amplitude. (**B**) In this cell, serotonin evokes a decrease in relative cell volume of 15% as measured intracellularly with a Corning K^+ exchanger-filled microelectrode after tetramethylammonium loading of the cell via bath application. [Figure A from K. Ballanyi, unpublished observations; Figure B from Ballanyi et al. (1990), with permission.]

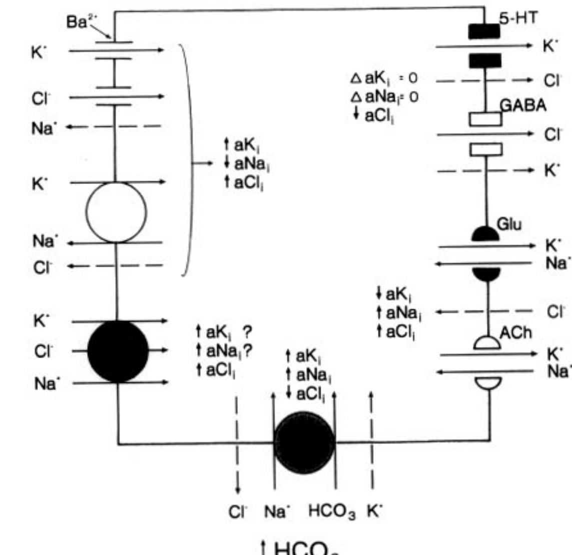


FIG. 18-8. The scheme summarizes the effects of elevated concentrations of extracellular potassium (K^+) and bicarbonate (HCO_3^-) as well as of the neurotransmitters serotonin (5-HT), γ-aminobutyric acid (GABA), glutamate (Glu) and acetylcholine (ACh) on glial activities of K^+, Na^+, and Cl^- (aK_i, aNa_i, aCl_i). Both 5-HT and GABA produce an equimolar efflux of KCl, which leads to a decrease of aCl_i, but not of aK_i ($\Delta aK_i = 0$) or aNa_i ($\Delta aNa_i = 0$) due to the concomitant cell shrinkage (see text). Both Glu and ACh, activate a nonspecific cation conductance, which results in an increase of aNa_i and a decrease of aK_i. The increase of aCl_i during the action of Glu and ACh can be explained by a passive redistribution of Cl^-. Secondary, passive ion movements are indicated by dotted lines. The left side of the scheme summarizes mechanisms, contributing to glial ion accumulation in response to elevated K^+. **Top**: Channel-mediated, passive KCl uptake by a Donnan-like redistribution, which is inhibited by the K^+ channel blocker Ba^{2+}. **Center** (*open circle*): K^+ uptake via the electrogenic Na^+-K^+ pump, which is blocked by ouabain. During profound neuronal activity, both these transport systems are active. **Bottom** (*filled circle*): Na^+-K^+-$2Cl^-$ cotransport, which is inhibited by furosemide, appears to be involved in maintenance of an aCl_i above equilibrium in some glial cells. Activation of electrogenic Na^+-HCO_3^- cotransport (*shaded circle*), which can be blocked by DIDS, can have long-term effects on aK_i, aNa_i, and aCl_i. Most of these ion movements are accompanied by water movements. (For details see text.)

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19 Gap junctions

BRUCE R. RANSOM

An intriguing form of intercellular communication is mediated by large channels called gap junctions that directly link the cytoplasmic interiors of cells (Loewenstein, 1981). Nearly every type of mammalian cell exhibits a degree of this form of interaction except mature skeletal muscle, spermatozoa, and erythrocytes (Dermietzel and Spray, 1993). Cellular coupling mediated by gap junctions is common in the nervous system, primarily among glial cells (Kuffler and Nicholls, 1967; Dermietzel and Spray, 1993). The characteristics and possible functions of these channels in glial cells are the focus of this review. As a necessary preliminary to discussing glial coupling, however, the general properties of gap junctional communication are surveyed.

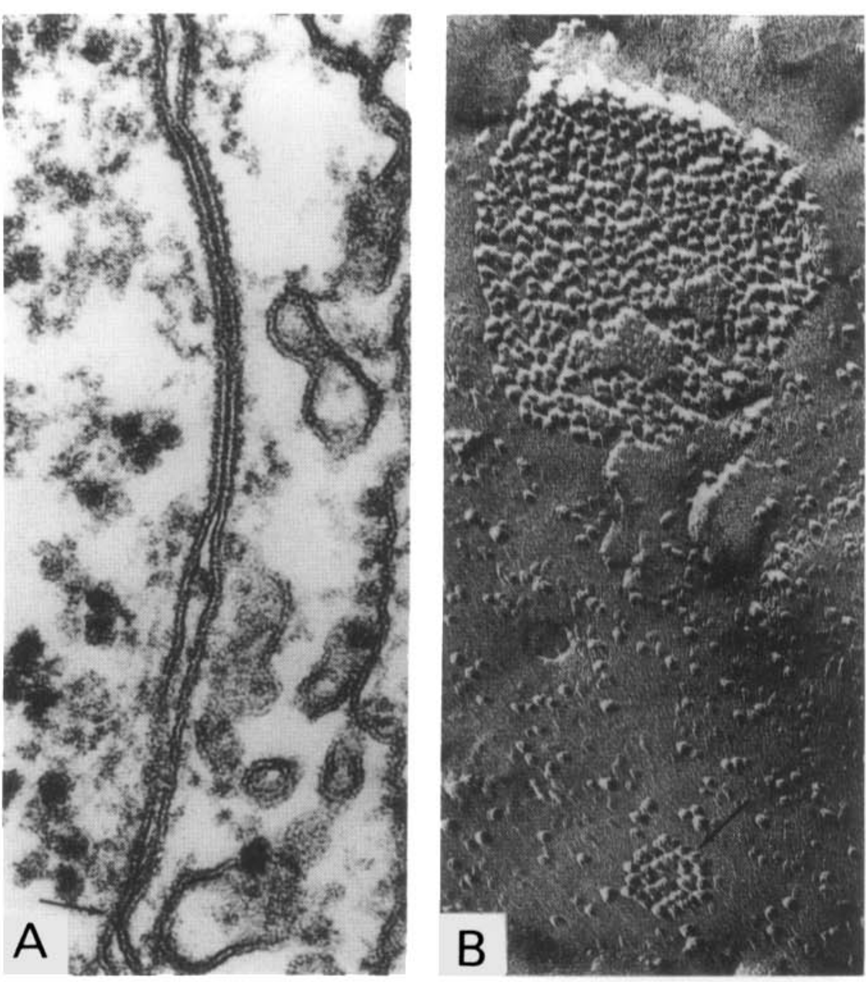
Direct intercellular coupling was first demonstrated in 1952 by Weidmann who showed that voltage changes in individual heart muscle cells were conveyed to neighboring cells (Weidmann, 1952). Electrical communication between neurons was demonstrated a few years later in the crayfish, representing the first example of an electrical synapse (Furshpan and Potter, 1959). Similar electrical communication was seen by Kuffler and his colleagues between glial cells in the leech central nervous system (Kuffler and Potter, 1964). How such electrical interactions might occur was not initially clear. The discovery by Kanno and Loewenstein (1964) that large molecules could pass between electrically coupled cells suggested the notion of a specific channel linking the cells. Shortly thereafter, the concept emerged that proteins formed membrane channels, and it was soon recognized that specialized membrane contacts mediated direct intercellular communication (Loewenstein, 1981); these contacts became known as gap junctions, based on electromicroscopic studies showing that at these sites adjacent membranes are more closely opposed, narrowing the extracellular space to a small "gap" (Revel and Karnowsky, 1967).

The channels comprising a gap junction are large-diameter aqueous pores that extend from one cell, across the extracellular space, into an adjacent cell. These channels allow all inorganic ions and small organic molecules to diffuse freely from the interior of one cell into the interior of another cell. The discovery of gap-junctional communication challenged the fundamental concept of classical cell theory, which states that individual cells are autonomous functional units. When cells are connected with their neighboring cells by gap junctions, the functional unit is no longer the individual cell of classical cell theory but rather the large aggregate of cells that are so joined, because they share so much of their cytoplasmic contents (Loewenstein, 1981). The strength of coupling between cells that form gap junctions with each other is highly variable, however, and the above statement is relevant primarily to strongly coupled cells.

GENERAL FEATURES OF GAP JUNCTIONS

Structure and Molecular Biology

In electron micrographs, gap junctions appear as discrete areas where the cell membranes of adjacent cells closely approach one another, leaving a narrow gap of only about 2 nm (Figures 19-1A and 19-2). These junctions have a seven-layer (septilaminar) appearance because a small layer of extracellular space can be seen between the three-layered cell membranes (Brightman and Reese, 1969) (Figure 19-2B). Using the freeze-fracture technique, a cluster of homogeneous intramembrane particles can be demonstrated on the cytoplasmic fracture face of the plasma membranes involved in the gap junction (Figure 19-1B). Each of these particles represents a gap junction hemichannel, and has been termed a *connexon* (Caspar et al., 1977). The diameter of the central hole in each connexon appears to be about 2 nm, with slight narrowing of the opening on the extracellular side of the membrane (Makowski, 1985) (Figure 19-1C). X-ray diffraction experiments indicate that each connexon hemichannel is composed of six symmetrical subunits (Makowski et al., 1977), but other investigators have observed that connexon particles are frequently composed of only 4 or 5 subunits (Hanna et al., 1985). Nevertheless, the most widely accepted model of a connexon



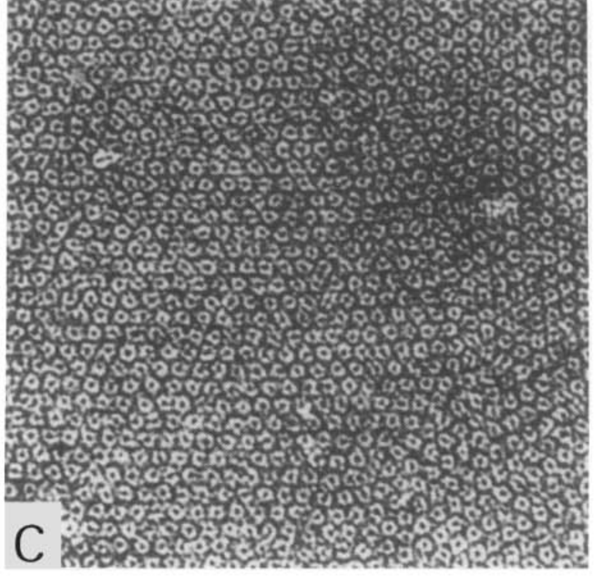


FIG. 19-1. Ultrastructural features of gap junctions between fibroblasts (A, B) and liver cells (C). (**A**) Transmission electron microscopy shows the characteristic close membrane apposition at sites where cells form gap junctions. A large junction and a small junction (*arrow*) are shown. Bar = ~200 μm. (**B**) Freeze fracture technique shows aggregates of membrane particles at gap junctions. Again, a large and a small (*arrow*) gap junction are shown. (**C**) Electron micrograph showing part of an isolated gap junction from rat liver. This oblique or *en face* view illustrates the tightly packed organization of the connexons which make up gap junctions. Each connexon has a densely stained center hole with a diameter of about 2 nm, which is presumed to be an aqueous pore. [Figures A and B from Gilula (1974) and Figure C from Gilula (1978), with permission.]

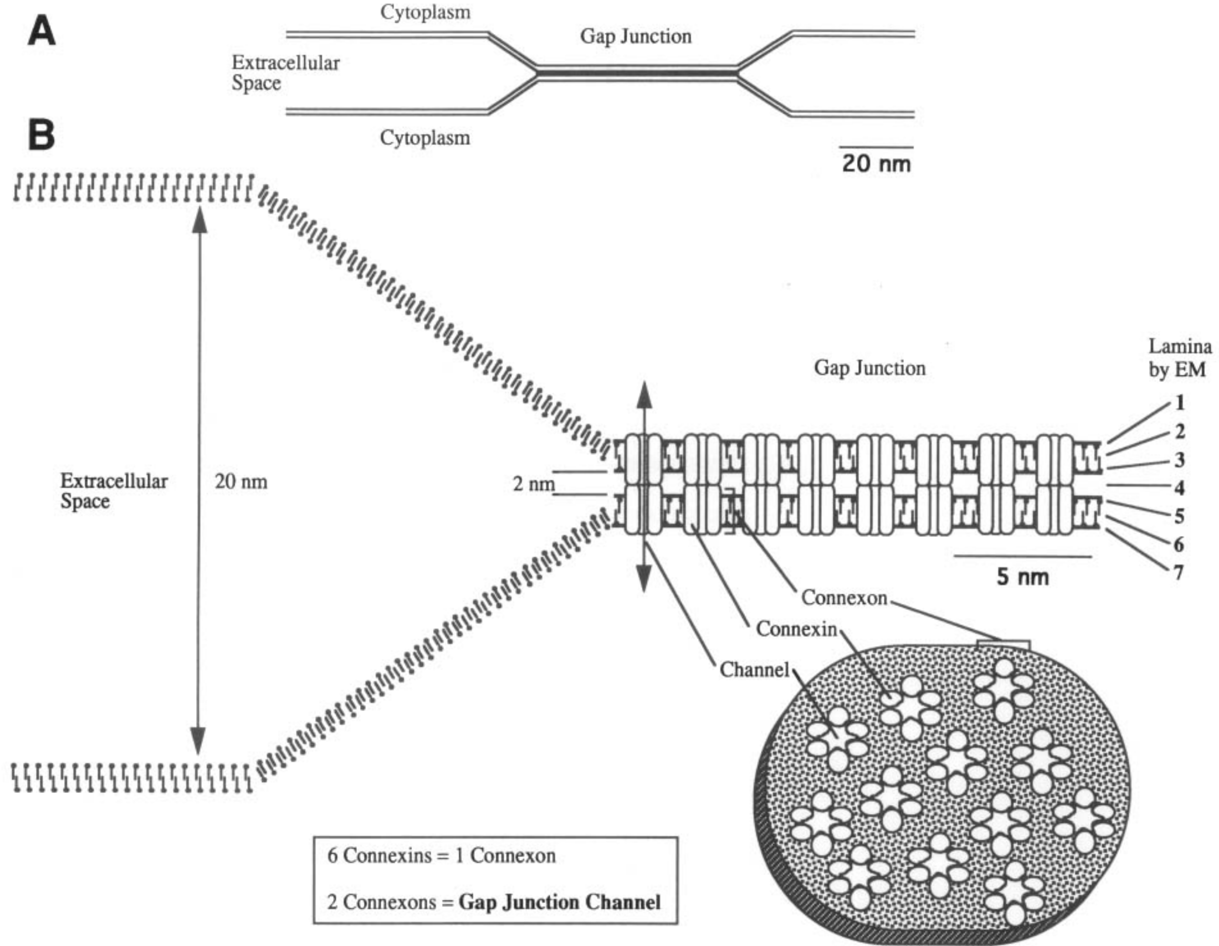
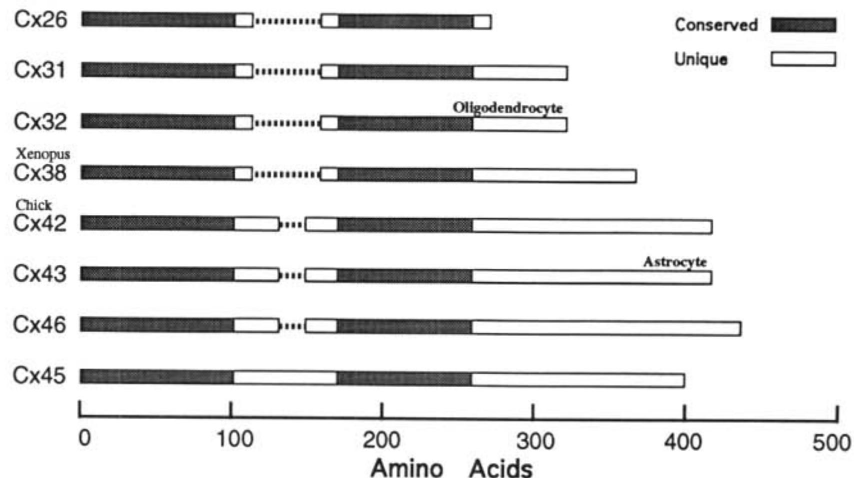


FIG. 19-2. Idealized diagram of a gap junction based on x-ray diffraction and electron microscope studies. (**A**) Low-power electron-microscopic appearance of a gap junction. The junction looks five-layered at this magnification. (**B**) At higher magnification, the small slit of extracellular space that remains between cells at the junction is easily seen and the structure looks seven-layered (septilaminar). The gap junction is believed to be composed of six identical connexin proteins which form hemichannels called connexons (Makowski et al., 1977). Two connexons connect with one another and bridge the extracellular space. The insert figure shows a section of gap junction in *en face* perspective, that is, as if the gap junction were separated in the middle and viewed from the extracellular side.

hemichannel is the aggregation of six identical protein molecules, called *connexins* (Goodenough, 1974) (Figure 19-2B). A gap junction channel is formed when a connexon in one cell comes into perfect alignment with a second connexon in an adjacent cell; exactly how this occurs is not understood (Bennett et al., 1991). Structural studies indicate that hundreds of functional gap channels may aggregate at a given gap junction.

Significant progress has been made in the isolation and characterization of the proteins that form gap junctions (Dermietzel et al., 1990; Bennett et al., 1991; Beyer, 1993). About a dozen putative connexin proteins have been identified in mammalian tissues and seem to arise from two distinct gene families (Haefliger et al., 1992). The two major subclasses of these proteins, based on similar gene structure and about 50% amino acid sequence homology, are typified by connexins derived from liver and heart (Figure 19-3A). The gap junction proteins derived from liver have molecular weights of 26 kD and 32 kD (i.e., connexin26 and connexin32) and the gap junction protein derived from heart has a molecular weight of 43 (connexin43) (Bennett, et al., 1991). These connexin proteins are distributed in a nonrandom way in mammalian tissues, including the brain (Dermietzel and Spray, 1993). The cellular pattern of connexin expression within the brain is becoming clear; neurons and oligodendrocytes express connexin32, astrocytes express connexin43, and ependyma and leptomeningeal cells express both connexin26 and connexin43. Connexin43, however, is more strongly expressed than any of the others (Dermietzel et al., 1989), which is consistent with



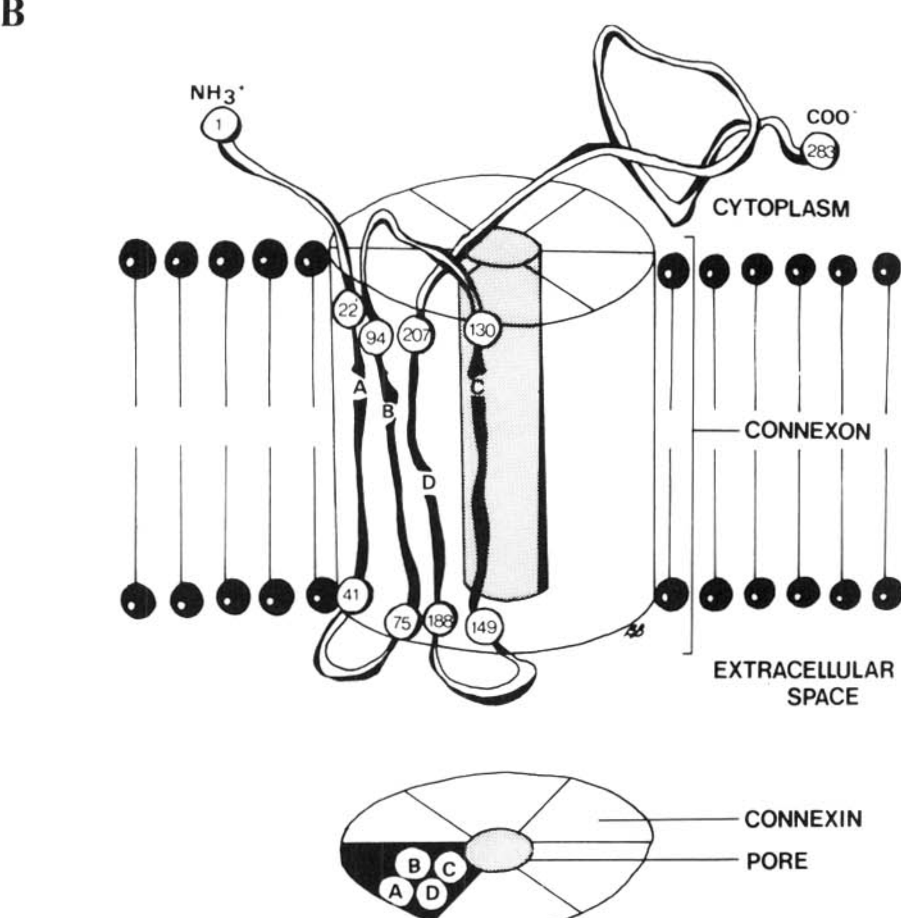


FIG. 19-3. Connexin proteins make up junctions. (**A**) The amino acid sequences of several cloned connexins are compared in this diagram. Regions where the amino acid sequences are highly similar are *shaded*. *Dashes* represent spaces added to optimize alignment. Evidence suggests that connexin32 and connexin43 make up gap junctions in oligodendrocytes and astrocytes, respectively. [Adapted from Beyer (1993), with permission.] (**B**) Diagram illustrating the manner in which an individual connexin is inserted into the membrane. There are four membrane spanning regions and the N terminus and C terminus are both located on the cytoplasmic side of the membrane. Six similar molecules are presumed to make up each connexon. [From Dermietzel et al. (1991), with permission.]

the fact that astrocytes show the strongest functional coupling (Kettenmann and Ransom, 1988; see "Astrocytes" below).

That connexins actually mediate functional intercellular communication is attested to by the following observations: (1) connexin mRNA can induce cell-cell channel formation (Dahl et al., 1987); (2) connexin protein reconstituted into artificial membranes produces channels that have the expected biophysical behavior (Spray et al., 1986); and (3) intracellular injection of antibodies against connexin blocks cell coupling (Hertzberg et al., 1985; Dudek et al., 1988). Moreover, strong evidence supports the corollary expectation that connexins form gap junctions. For example, poorly coupled glioma cells that do not express gap junctions can be induced, by transfection with connexin43 cDNA, to express both gap junctions and coupling; the gap junctions are specifically marked by antibodies to connexin43 (Naus et al., 1993).

The manner in which connexins are inserted into membranes has been deduced on the basis of hydropathicity plots and via the use of site-specific antibodies and proteolytic degradation (Hertzberg et al., 1988; Dermietzel et al., 1990), and a plausible model is shown in Figure 19-3B. Because there are four hydrophobic amino acid sequence domains, it is presumed that the protein crosses the membrane four times. Both the N terminus and the C terminus of connexin proteins are believed to lay on the cytoplasmic side of the membrane, resulting in two extracellular and one intracellular loop. The greatest degree of homology between connexin proteins occurs in the membrane spanning regions and in the two extracellular loops, while substantial variability is noted in the length and amino acid composition of the cytoplasmic sections, especially the C terminus (Bennett et al., 1991). The protein segments that form the walls of the aqueous channel may be more hydrophilic than the other membrane-spanning regions and, perhaps, have a slight excess of negative charges explaining the tendency toward reduced permeation by large negatively charged molecules (Neyton and Trautwein, 1985).

It is well established that nonsimilar cells, that is, from different organs or different species, may form gap junctions with one another (Loewenstein, 1981; Bennett et al., 1991). This observation suggested, and subsequent experiments have proven, that connexon hemichannels of different protein composition may form functional gap junction channels with one another. Details about the biophysical function of these heterologous channels are still forthcoming, but such channels may have unique permeability characteristics (Robinson et al., 1993; see "Oligodendrites" below).

Biophysical Properties

The minimal diameter of the gap junction channel in vertebrates is about 1.6 nm (Schwarzmann et al., 1981). These pores are permeable to molecules up to a molecular weight of approximately 1 kD, largely independent of charge (Loewenstein, 1981). As a consequence, coupled cells are able to exchange a wide range of biologically important molecules, including cyclic nucleotides, small molecules of intermediary metabolism, vitamins, and inorganic ions (Saez et al., 1989). This does not necessarily imply that coupled cells will have identical concentrations of all permeant molecules; the concentration of a given permeant molecule in each cell would depend on the transmembrane fluxes, and production and removal of the molecule in question, in addition to the gap junctional permeability of that molecule (Neyton and Trautwein, 1985; Bennett et al., 1991). Obviously, the tendency for coupled cells to have the same concentration of a cytoplasmic constituent will increase with the strength of coupling.

Electrophysiological analysis of single gap junction channels indicate that they abruptly open and close in a manner similar to that of other ion channels (Neyton and Trautwein, 1985; Bennett et al., 1991). The conductance of individual gap junction channels has been measured and appears to vary with the type of connexin protein from which the channel is constructed. The unitary conductance for connexin32 channels is about 120 to 150 pS, and for connexin43 channels this value is about 50 pS (Bennett et al., 1991). Questions remain about the possible existence of subconductance states, the extent to which single-channel conductance might be modified by membrane environment, and the biophysical characteristics of channels composed of other connexin proteins. Given the fact that gap junction channels are permeable to much larger molecules than conventional ion channels, it is surprising that the unitary conductance of gap junction channels is actually smaller than some ion channels such as the "big K" (BK) Ca^{2+}-activated K^+ channel (i.e., 200 to 300 pS) (Blatz and Magelby, 1987). One explanation may be the simple fact that the gap junction channel is physically longer than an average ion channel because it spans two membrane bilayers instead of one (Bennett et al., 1991); this would increase the channel's total electrical resistance, that is, decrease its conductance.

The permeability of gap junction channels is influ-

enced by a number of physiological variables, primarily transjunctional voltage, and the intracellular concentrations of H^+ and Ca^{2+}. The exact mechanisms by which these factors "gate" conductance are not known, but Unwin (1989) has proposed an operational model based on electron-microscopic studies. Under conditions where the channel would be expected to be closed, the protein subunits of the channel appear tilted. It is hypothesized that in the tilted state the alignment of the protein segments forming the walls of the channel pore is altered in such a manner that some of their constituent amino acids move into a channel-blocking position.

The voltage sensitivity of gap-junctional conductance depends on the type of connexin protein that forms the junction. Junctions formed by connexin32, the connexin primarily expressed by oligodendrocytes (Dermietzel et al., 1989), are closed by transjunctional voltage gradients of either sign, but the degree of sensitivity is low (Bennett et al., 1991). The connexin protein making up astrocytic gap junctions, connexin43, is less voltage-sensitive (Dermietzel et al., 1991). Increases in intracellular concentrations of either H^+ or Ca^{2+} rapidly decrease junctional conductance (Loewenstein, 1981; Spray et al., 1981). Coupling conductance is sensitive to changes in intracellular pH that are within the physiological range of this variable (i.e., 6.5 ± 0.5 pH; Bennett et al., 1988). The increases in intracellular Ca^{2+} that result in decreased junctional conductance are high (e.g., ≥10 μM) and this variable is not likely to affect coupling under normal physiological conditions (DeMello, 1982; but see Lazrak and Peracchia, 1993); cell injury causes large increases in intracellular [Ca^{2+}], which could lead to uncoupling. Lipophilic agents such as the higher order alcohols heptanol and octanol, and the anesthetic halothane, block gap junction channels (Spray and Bennett, 1985) but the mechanism of blockade by these agents is not known. The sensitivity of junctional conductance to octanol varies considerably among astrocytes from different brain regions (Lee et al., 1994), even though they would be expected to express the same connexin protein.

Second messengers have complex effects on gap-junctional conductance (Bennett et al., 1991). For example, elevation of cyclic adenosine monophosphate (cAMP) increases junctional conductance in hepatocytes and myocytes, expressing connexin32 and -43, respectively, while decreasing the gap junction conductance of uterine muscle cells, also expressing connexin43. These changes occur within minutes, suggesting phosphorylation or effects on channel gating. In other systems, an increase of cAMP increases junctional conductance over a period of hours; this effect can be prevented by blocking mRNA or protein synthesis, suggesting that channels are being newly synthesized. It is worth noting that the turnover of connexin proteins appears to be rapid, with protein lifetimes of several hours (Bennett et al., 1991). These second-messenger effects, which may be initiated by a wide range of agents, including hormones and neurotransmitters, imply an important degree of plasticity in the control of physiological events mediated by gap-junctional communication. Several excellent reviews may be consulted for more detailed general information about the molecular biology and biophysics of gap junctions (Loewenstein, 1981; Bennett et al., 1991; Dermietzel et al., 1991; Beyer, 1993).

GAP JUNCTIONS IN GLIAL CELLS

Distribution and Anatomy

Gap junctions are found in most mammalian glial cells (Brightman and Reese, 1969; Mugnaini, 1986; Peters et al., 1991). Only microglial cells seem devoid of these junctions. Mammalian Müller cells, specialized glia of the retina (Chapter 4, this volume), do not exhibit gap junctions except *in vitro* (Uga and Smelser, 1973; Wolburg et al., 1990); nonmammalian Müller cells, however, express them (Mobbs et al., 1988). The electron-microscopic and freeze-fracture features of glial gap junctions are similar to what has been described in other cells (Mugnaini, 1986; Peters et al., 1991) (see Figure 19-1). The ultrastructural demonstration of gap junctions between cells is well correlated with the presence of functional intercellular communication between them (Loewenstein, 1981), but ultrastructural analysis alone can not define the strength or pattern of physiological cell coupling.

Astrocytes of various types (i.e., protoplasmic, fibrous, Bergmann cells) form numerous gap junctions with other astrocytes (Brightman and Reese, 1969; Dermietzel, 1974; Massa and Mugnaini, 1982; Mugnaini, 1986), and with oligodendrocytes (Massa and Mugnaini, 1985; Mugnaini, 1986) and ependymal cells (Mugnaini, 1986). The extent to which gap junctions form between fibrous (primarily in white matter) and protoplasmic astrocytes (primarily in gray matter) is unclear (Mugnaini, 1986). Type-2 astrocytes of the rat optic nerve (following the nomenclature of Raff, 1989) do not show coupling *in vitro* (Sontheimer et al., 1990), suggesting the possibility that not all astrocytes are coupled to one another (see below). Gap junctions are expressed by

astrocytes making up human brain tumors (i.e., astrocytomas), but are markedly decreased in the most malignant type of astrocyte tumor, glioblastoma multiforme (Tani et al., 1973). Considerable evidence suggests that the loss of gap-junctional communication may be associated with neoplasia (Loewenstein, 1981; Klaunig and Ruch, 1990).

Few, if any, gap junctions are found between oligodendrocytes in white matter of the cat, although gap junctions are abundant between oligodendrocytes and astrocytes (Massa and Mugnaini, 1982; Mugnaini, 1986). It was proposed that communication between oligodendrocytes is only possible via their connections with the astrocytic syncytium (Massa and Mugnaini, 1985). The generality of this scheme, however, is questioned by the demonstration of gap junctions between purified oligodendrocytes from rat (Gonatas et al., 1982) and cow (Wollman et al., 1981), and the finding of functional coupling between oligodendrocytes (i.e., without communication through astrocytes) *in vitro* (Kettenmann and Ransom, 1988; Blankenfeld et al., 1993; Ransom and Kettenmann, 1990) and *in situ* (Berger et al., 1991; Butt and Ransom, 1993; Robinson et al., 1993).

Nonmyelinating Schwann cells *in vitro* exhibit dye coupling, which disappears when they begin to make myelin (Konishi, 1990); gap junctions between these cells have not been reported, but this may simply be for want of a properly timed ultrastructural study. Enteric glia express gap junctions (Gabella, 1981) and robust intercellular coupling (Hanani et al., 1989). Ependymal cells form gap junctions with each other and with astrocytes (Brightman and Reese, 1969; Mugnaini, 1986). The specialized glial cells of the intermediate lobe of the pituitary, called *stellate glia,* exhibit gap junctions and functional coupling (Mudrick-Donnon et al., 1993).

Dye and Electrical Coupling

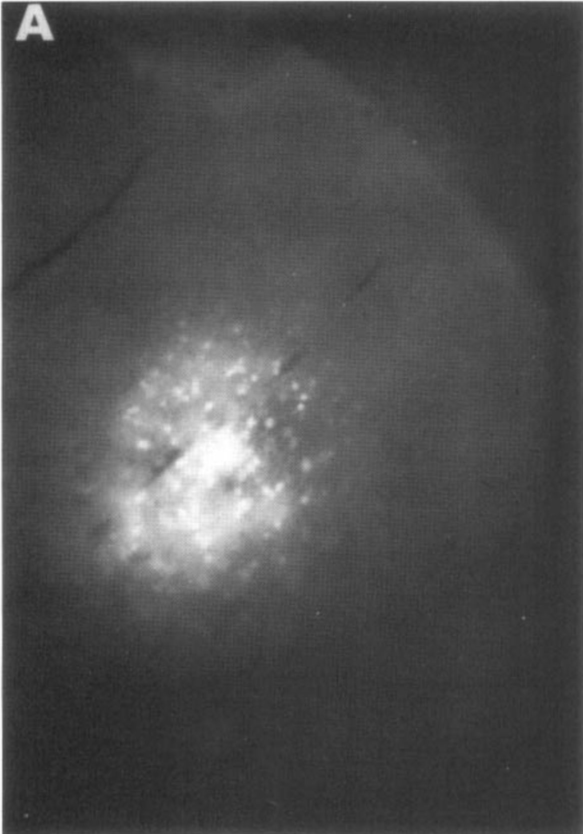
The possibility that mammalian glial cells might be coupled to one another was suggested by indirect electrophysiological observations; the closed field distribution of glial-generated cortical slow potentials resulting from electrical stimulation (Ransom, 1974; Somjen, 1970), the low input resistance of cortical glia (Trachtenberg and Pollen, 1970), and the depolarization of glial cells in sites within the cortex that are distant from focal accumulations of K^+ (Futamachi and Pedley, 1976), all imply that mammalian glia form an electrical syncytium. Subsequently, coupling between glial cells has been studied in three ways:

1. A junction-permeable fluorescent dye such as lucifer yellow is injected into individual cells to see if it diffuses into adjacent cells (Ransom and Sontheimer, 1992). This technique provides primarily qualitative information.
2. Microelectrodes are placed in two nearby cells to determine if current injected into one cell passes into the other cell (e.g., Kettenmann and Ransom, 1988). The strength of coupling can be quantified by measuring the voltage or current that is transferred between the two cells.
3. The newest method for measuring coupling uses laser light to bleach and destroy the fluorescent dye molecules in an individual cell; strength of coupling to adjacent cells is judged by the speed with which dye diffuses back into this cell from nearby cells via gap junctions (Sontheimer, 1992). This method is termed laser photobleaching.

Nonmammalian Glial Cells. Direct electrical communication between nonmammalian glial cells, was firmly established by Kuffler and his colleagues in studies on the leech central nervous system (Kuffler and Potter, 1964) and the mudpuppy optic nerve (Kuffler et al., 1966; Cohen, 1970). Conventional gap junctions appear to mediate coupling between glial cells in the leech (Coggeshall, 1974). The possibility of coupling between neurons and glial cells was directly tested in the leech, and these two cell types were not coupled (Kuffler and Potter, 1964). Glial cells in the mudpuppy optic nerve show strong dye-coupling and this is blocked by cytoplasmic acidification (Tang et al., 1985).

In fish, in contrast to invertebrates, macroglial cells are divisible into astrocytes and oligodendrocytes (Diaz-Regueira et al., 1992). Gap junctions are seen between the somata of oligodendrocytes and between the processes of astrocytes (Diaz-Regueira et al., 1992), and fish astrocytes are dye-coupled *in vitro* (Hoppe et al., 1991).

Astrocytes. Mammalian astrocytes usually, but not always, show widespread dye-coupling with each other. When lucifer yellow is injected into individual cortical astrocytes as many as 100 adjacent cells are stained (Figure 19-4) (Gutnick et al., 1981; Connors et al., 1984; Binmöller and Müller, 1992). The coupled cells form spherical arrays, suggesting that lucifer yellow diffuses from the injected astrocyte into uniformly coupled surrounding cells. All of the cells in the coupled arrays have a similar appearance, but only the injected cell could be clearly evaluated as to cellular detail (Figure 19-4B); these cells have the appearance of protoplasmic astrocytes (Gutnick et al., 1981). White matter astrocytes are also highly



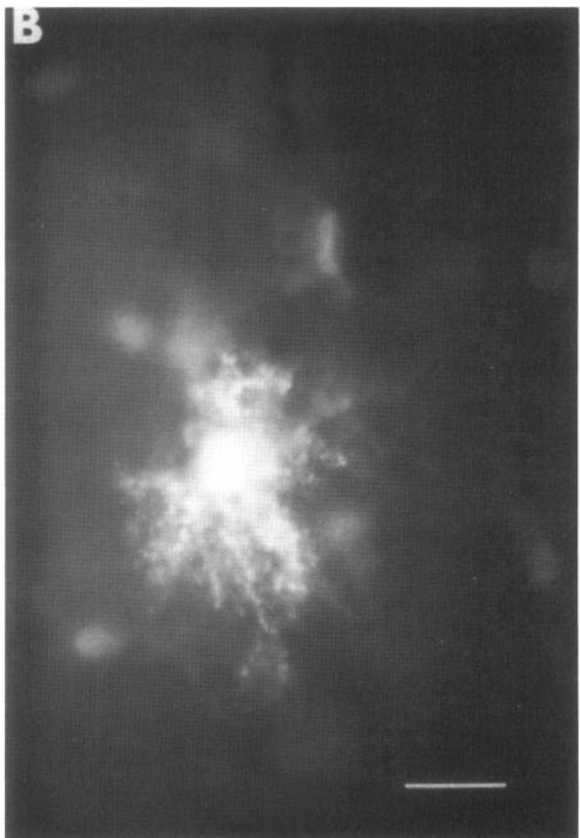


FIG. 19-4. Fluorescent dye coupling between astrocytes in guinea pig neocortical brain slice. (**A, B**) Photomicrographs of the same field at different magnifications. A single cell, identified by physiological criteria as a glial cell, was injected with lucifer yellow (*LY*). The central, brightly stained cell, has characteristics of a protoplasmic astrocyte (*B*), and is surrounded by numerous, less intensely stained cells with similar features (*A* & *B*). Scale: 200 μm in Figure A, 50 μm in Figure B. [Adapted from Gutnick et al. (1981), with permission.]

dye-coupled *in situ* (Figure 19-5A–C) (Butt and Ransom, 1989; Butt and Ransom, 1993). *In vitro* studies of white matter astrocytes, derived from optic nerve, indicate that dye-coupling is restricted to type-1 astrocytes, with type-2 astrocytes devoid of coupling to each other or to the type-1 cells (Sontheimer et al., 1990); consistent with this observation, only the type-1 cells express connexin43 (Belliveau and Naus, 1994). Presently, however, the existence of a unique astrocyte in the mammalian central nervous system with characteristics similar to the type-2 astrocyte defined *in vitro*, is controversial (Fulton et al., 1991). Moreover, studies of astrocyte dye-coupling *in situ* have thus far failed to detect subpopulations of non-coupled astrocytes (Butt and Ransom, 1993). Surprisingly, cat neocortical astrocytes studied *in vivo* did not exhibit dye-coupling (Takato and Goldring, 1979). Failure to see dye-coupling among these cells may have related to technical aspects of dye injection (see Gutnick et al., 1981) or possibly to a state of unintentional respiratory acidosis.

Astrocytes grown *in vitro* are not always dye-coupled. A maximum of ~50% of hippocampal astrocytes (Sontheimer et al., 1991) and ~80% of optic nerve astrocytes (Sontheimer et al., 1990) are coupled. It should be borne in mind, however, that weak electrical coupling can occur without dye-coupling (e.g., Ransom and Kettenmann, 1990). In hippocampal astrocytes, the expression of Na^+ channels and the existence of dye-coupling are mutually exclusive (Sontheimer et al., 1991). Astrocytes require a nominal concentration of intracellular Na^+ for survival and Na^+ influx via Na^+ channels may provide for this (Sontheimer et al., 1994); perhaps this function is also provided by coupling, which would allow cells with Na^+ influx via other routes (Na^+-dependent transporters, etc.) to share their intracellular Na^+ with neighboring cells, obviating the need for Na^+ channels.

In the rodent central nervous system, astrocytes proliferate and differentiate in the early postnatal period (Skoff et al., 1976). Dye-coupling in both gray

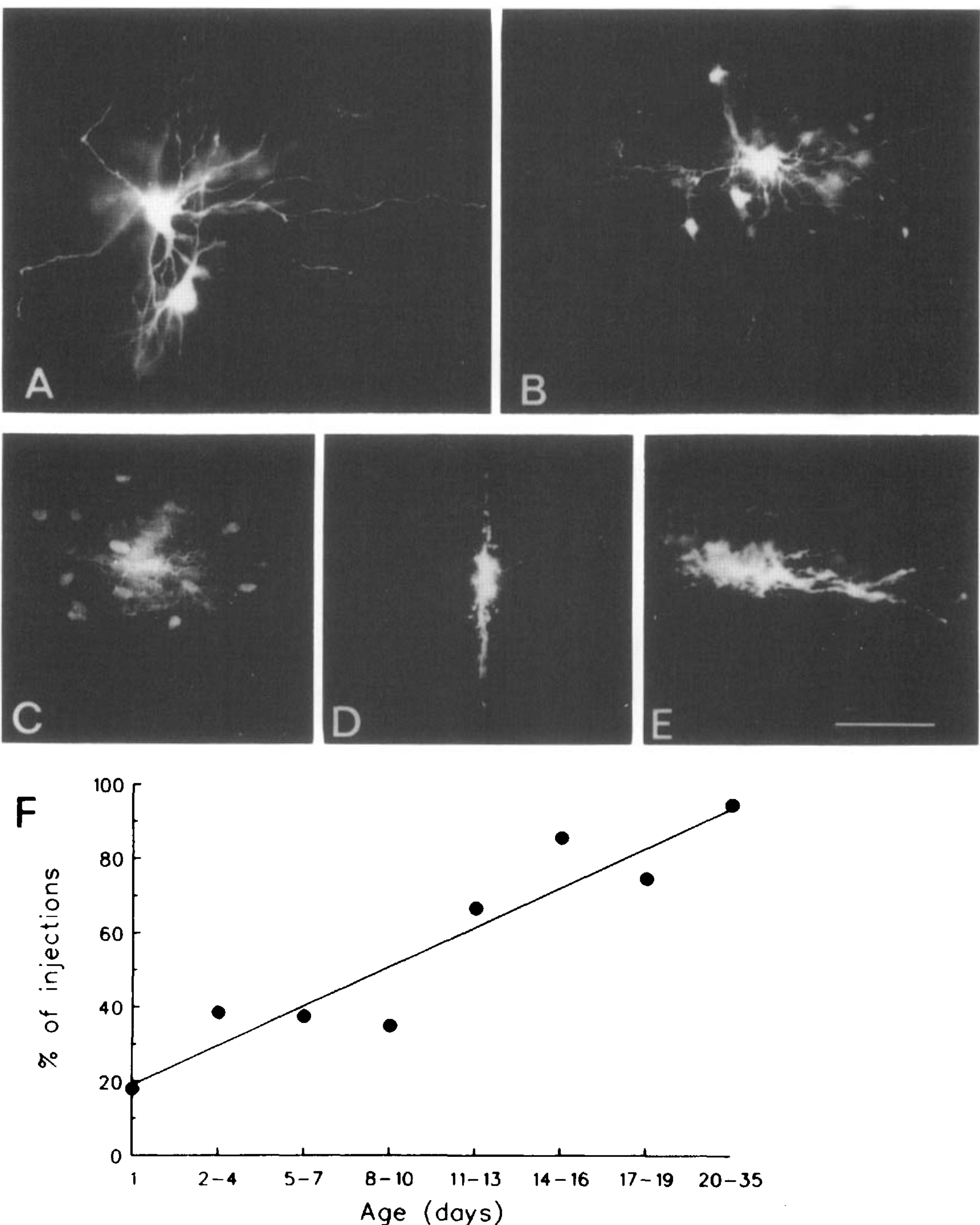


FIG. 19-5. Dye coupling in LY-filled astrocytes in white matter. Individual astrocytes in the intact rat optic nerve were injected with LY. (A, B) Mature astrocytes in the optic nerve are almost always dye coupled, occasionally to just a few cells (A), but usually to a large number of cells (B, C). Solitary astrocytes were stained following treatment with 1 μM octanol (D) or 50% CO_2 (E); these procedures are known to uncouple gap junctions. Bar in E indicates long axis of the nerve and is equal to 20 μm for A; 50 μm for B and C; and 70 μm for D and E. (F) Dye coupling between astrocytes increases with development, and 95% of LY-filled astrocytes in the mature nerve are coupled. This graph shows the percent of cell injections which resulted in multiple cell filling, as a function of postnatal age. [From Butt and Ransom (1993), with permission.]

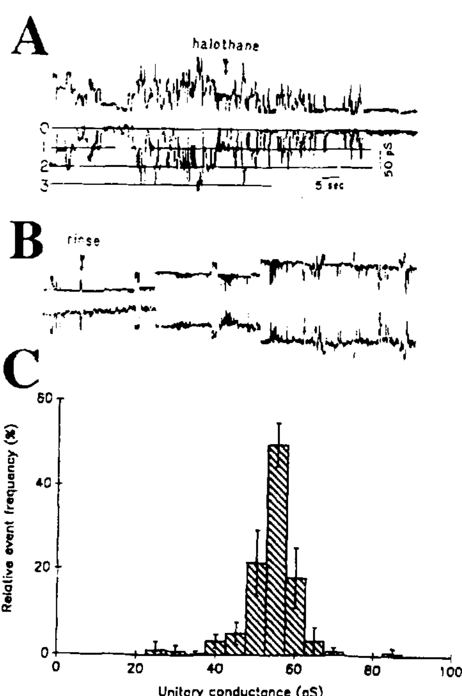
matter and white matter astrocytes develops as the cells mature, and this is seen both *in vivo* and *in vitro* (Sontheimer et al., 1990; Sontheimer et al., 1991; Binmöller and Müller, 1992; Butt and Ransom, 1993) (Figure 19-5F). The distribution of connexin43, the protein molecule that forms astrocyte gap junctions, likewise appears to increase in distribution postnatally (Dermietzel et al., 1989). This is in contrast to rat cortical neurons. These cells show intense coupling, in columnar aggregates, during early development and this progressively disappears with maturation (Lo Turco and Kreigstein, 1991).

Intercellular communication between astrocytes has been studied more quantitatively using electrophysiological methods. Most studies have used cultured astrocytes because of the technical advantage of being able to record from cell pairs under direct visual control. Rat cortical astrocytes in confluent monolayer cultures are strongly coupled electrically (Kettenmann and Ransom, 1988). All astrocytes within 300 μm of one another are coupled and the average coupling ratio (i.e., voltage change in the current-injected cell divided by the voltage change in a nearby cell) of astrocytes within 100 μm of each other is 0.44. Application of 0.5 mM Ba^{2+} or 44 mM Cs^+, ions that block K^+ conductance, depolarizes astrocytes, increases their input resistance, and dramatically increases coupling ratio; the coupling ratio is believed to increase due to an increase in the effective length constant of the glial membrane without any direct effect on junctional resistance (Kettenmann and Ransom, 1988).

The greater resolving power of the patch-clamp recording technique has been applied to the analysis of gap junctional communication between astrocytes. Pairs of adjacent astrocytes are voltage-clamped using the double whole-cell recording technique allowing the resolution of single gap junction-channel current events; the unitary conductance of single channels between astrocytes is 50 to 60 pS (Dermietzel et al., 1991; Giaume et al., 1991a) (Figure 19-6A–C). Cell pairs studied within hours after being dissociated from confluent glial cultures were strongly coupled to one another (average junctional conductance = 13 mS; Dermietzel et al., 1991). Based on a unitary junctional conductance of about 55 pS, these pairs of astrocytes appeared to be connected by approximately 235 functional gap junction channels. Gap-junctional conductance was relatively insensitive to transjunctional voltage over a range of ±50 mV (Dermietzel et al., 1991; see also Kettenmann and Ransom, 1988) but did diminish at higher transjunctional voltages (Giaume et al., 1991a). Junctional conductance was diminished by heptanol (1.5 mM), octanol (1 mM), elevated CO_2 and halothane (2 mM) (Connors et al., 1984; Dermietzel et al., 1991; Giaume et al., 1991a; Butt and Ransom, 1993). (Figure 19-5D, E and 6A, B) The ease with which octanol uncouples astrocytes appears to depend on the brain region from which the cells were derived (Lee et al., 1994); spinal cord astrocytes were the most sensitive and hippocampal astrocytes the least. In general, astrocyte gap junctions behaved similarly to gap junctions studied in other tissues where junctions are formed by connexin43 (Spray and Burt, 1990).

Within the brain, connexin43 appears to be expressed solely in astrocytes (Dermietzel and Spray, 1993). The concentration of connexin43 in the brain peaks in the early postnatal period and remains in high concentration thereafter. Strong evidence indicates that connexin43 is responsible for forming gap junctions in astrocytes (Dermietzel et al., 1989; Giaume et al., 1991a; Yamamoto et al., 1991; Dermietzel et al., 1992). The most convincing data derive from tissue culture experiments where astrocytes exhibiting functional intercellular communication express only connexin43 (Dermietzel et al., 1991; Giaume et al., 1991a). Connexin43 appears to be distributed in a plaquelike manner between astrocytes, as well as being located in the cytoplasm (Figure 19-6D, E). Ultrastructural analysis shows that connexin43 is, indeed, associated with the gap junctional complex itself (Dermietzel et al., 1991; Giaume et al., 1991a). A possible discrepancy in the association of connexin43 with astrocytic coupling has arisen with reactive astrocytes in the kainate-lesioned hippocampus. These cells exhibit robust dye-coupling (Burnard et al., 1990), in spite of the fact that connexin43 is apparently reduced in kainate-induced areas of gliosis (Vukelic et al., 1991).

Connexin43 appears to be widely distributed throughout the mammalian brain, although some areas show a higher concentration of the protein than others (Batter et al., 1992; Nagy et al., 1992). The concentration of connexin43 protein, cellular immunoreactivity and mRNA expression is four times greater in cultured astrocytes derived from hypothalamus compared to striatum. Furthermore, the hypothalamic astrocytes are more highly coupled than those from the striatum (Batter et al., 1992; see also Lee et al., 1994). Connexin43 is least expressed in spinal white matter (Nagy et al., 1992). The ratio of phosphorylated to nonphosphorylated forms of connexin43, believed related to the functional state of the protein, is constant from region to region (Batter et al., 1992; Nagy et al., 1992). These findings suggest that the strength of astrocyte intercellular communication varies with brain region and that this difference is controlled at



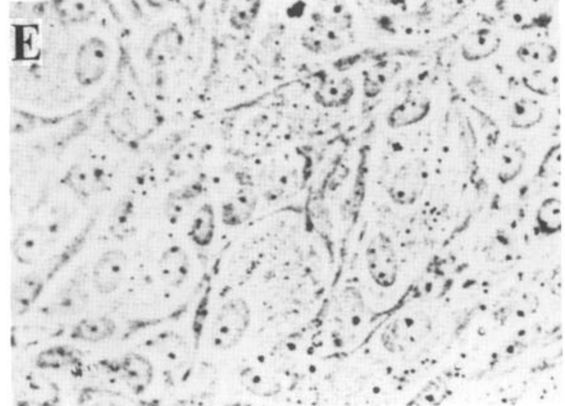


FIG. 19-6. Electrophysiological and molecular characteristics of rat forebrain astrocytes in culture. Pairs of astrocytes are recorded from using the double whole-cell recording technique. The two traces show the junctional current recorded in each astrocyte and the abrupt fluctuations represent openings and closings of individual gap junction channels. Voltage of the upper cell of the pair is held at 0 mV and voltage of the lower cell at −50 mV. Calibration bar represents 2.5 pA and corresponds to 50 pS. Application of halothane (1 μM) causes a decline in junctional current, which is reversible when the halothane is removed (B). (C) The frequency distribution of the unitary conductances of single gap junction channels is shown. The vast majority of these unitary conductances fall between 50 and 60 pS. (D) Immunohistochemical localization of anticonnexin43 immunoreactivity in 3-week-old astrocyte cultures derived from rat cortex and striatum. The connexin43 protein appears to be located in plaquelike areas between cells (*arrows*) as well as within the cytoplasm (*asterisks*). (E) A phase-contrast micrograph shows the cells in "D". ×700. [From Dermietzel *et al.* (1991), with permission.]

the level of gene expression. The functional significance of regional differences in astrocyte coupling, in the larger context of brain function, is not known.

Norepinephrine, apparently acting through a second-messenger pathway, downregulates functional gap junctional communication between striatal astrocytes (Giaume et al., 1991b). The norepinephrine effect may be mediated by activation of phospholipase C. Elevating cAMP by application of the beta-adrenergic agonist isoproterenol enhances dye-coupling (Giaume et al., 1991b). It was recently reported that astrocyte dye-coupling is increased by application of glutamate or high [K^+] solutions (Enkvist and McCarthy, 1994); brief applications of high [K^+] solutions did not increase coupling between oligodendrocytes and astrocytes (Ransom and Kettenmann, 1990). Glutamate or high [K^+] solution cause membrane depolarization and this might be linked to changes in coupling via changes in intracellular pH (Ransom, 1992). Depolarization produces an alkaline shift in astrocytes that could enhance coupling (Ransom, 1992). The effects of pH on coupling can be persistent; intracellular acidification mediated by lactate results in irreversible uncoupling if the acidification persists for thirty minutes (Anders, 1988).

Oligodendrocytes. Myelinating oligodendrocytes in situ, typically (i.e., 75% of fills) show dye-coupling to other oligodendrocytes (Butt and Ransom, 1989; Butt and Ransom, 1993) (Figure 19-7). The number of dye-coupled cells is small, usually between two and four, and coupling appears to occur at contacts

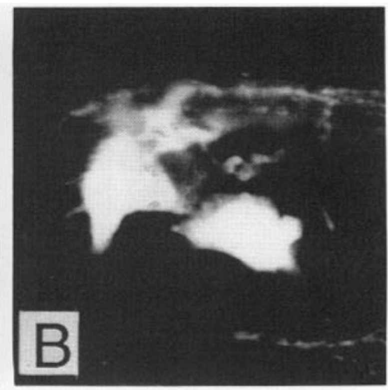


FIG. 19-7. Dye-coupling between mature oligodendrocytes in the intact rat optic nerve. Injection of LY into individual oligodendrocytes frequently resulted in the staining of two or more cells as seen here (A). The dye-coupled cells are easily recognized as oligodendrocytes because of their unique longitudinal processes (see Ransom et al., 1991). At higher magnification, closely adjacent dye coupled cell bodies can be seen (B). Bar in A indicates long axis of the nerve and = 50 μm in A and 20 μm in B. [From Butt and Ransom (1993), with permission.]

between adjacent cell bodies (Butt and Ransom, 1993). Oligodendrocytes in the rabbit retina also show dye-coupling (Robinson et al., 1993), but immature oligodendrocytes in mouse corpus callosum are not dye-coupled (Berger et al., 1991), perhaps related to their immaturity. Immature oligodendrocytes rarely show dye- or electrical coupling, but coupling gradually increases in frequency as the cells mature (Figure 19-8C) (Blankenfeld, et al., 1993).

Oligodendrocytes derived from mouse spinal cord and studied *in vitro,* show electrical coupling in about 75% of cell-pairs tested (Kettenmann et al., 1983; Kettenmann and Ransom, 1988). The coupling between oligodendrocytes is less intense than between astrocytes; coupled oligodendrocytes within 100 μm of each other have an average coupling ratio of 0.11. As with astrocytes, the application of either Cs^+ or Ba^{2+} enhances electrical coupling and allows it to be recognized between cell-pairs that were judged not to be coupled in control solution (Kettenmann and Ransom, 1988) (Figure 19-8D). Oligodendrocytes therefore appear to be widely coupled by junctions that allow only weak electrical interaction, in accordance with the ultrastructural observation that interoligodendrocytic gap junctions are much less frequent than gap junctions between astrocytes (Massa and Mugnaini, 1985).

Using the double whole-cell recording technique, the mean junctional conductance between coupled mouse oligodendrocytes is 2.7 nS (Blankenfeld et al., 1993) (Figure 19-8A, B). If each gap channel between oligodendrocytes has a mean conductance of 120 or 150 pS (Dermietzel et al., 1993; see below), the estimated number of channels forming junctions between oligodendrocytes would range from 10 to 30. Electrical coupling between oligodendrocytes is reversibly blocked by intracellular acidification (Kettenmann et al., 1990). To achieve uncoupling, however, intracellular pH has to be decreased to below 6.5 and this could only be achieved if the dominant pH_i regulating mechanism in these cells, the Na^+-HCO_3^- cotransporter, was blocked (Kettenmann et al., 1990).

In contrast to astrocytes, oligodendrocytes appear to express connexin32 *in situ* (Dermietzel et al., 1989). The level of connexin32 expression is less than that of connexin43 consistent with the less abundant gap junctions between oligodendrocytes (e.g., Massa and Mugnaini, 1985) and their weaker functional coupling (Kettenmann and Ransom, 1988). Details regarding the single-channel characteristics between oligodendrocytes are not yet available, but, based on the behavior of connexin32 in expression systems, it is likely that single-channel conductance is ~120 to 150 pS. These channels show somewhat greater sensitivity to transjunctional voltage than channels formed from connexin43 (Bennett et al., 1991).

Gap junctions are frequently observed ultrastructurally between astrocytes and oligodendrocytes (Massa and Mugnaini, 1985; Mugnaini, 1986). Consistent with this, intercellular coupling between astrocytes and oligodendrocytes has been demonstrated *in vitro* (Ransom and Kettenmann, 1990) and *in situ* (Robinson et al., 1993). In comparison to the strength of coupling between astrocytes and between oligodendrocytes, electrical coupling between astrocytes and oligodendrocytes is weak (Ransom and Kettenmann, 1990) (Figure 19-9). The average coupling ratio for cells within 100 μm of one another is 0.04. Under optimal conditions for the detection of coupling (i.e., in the presence of Ba^{2+} or Cs^+

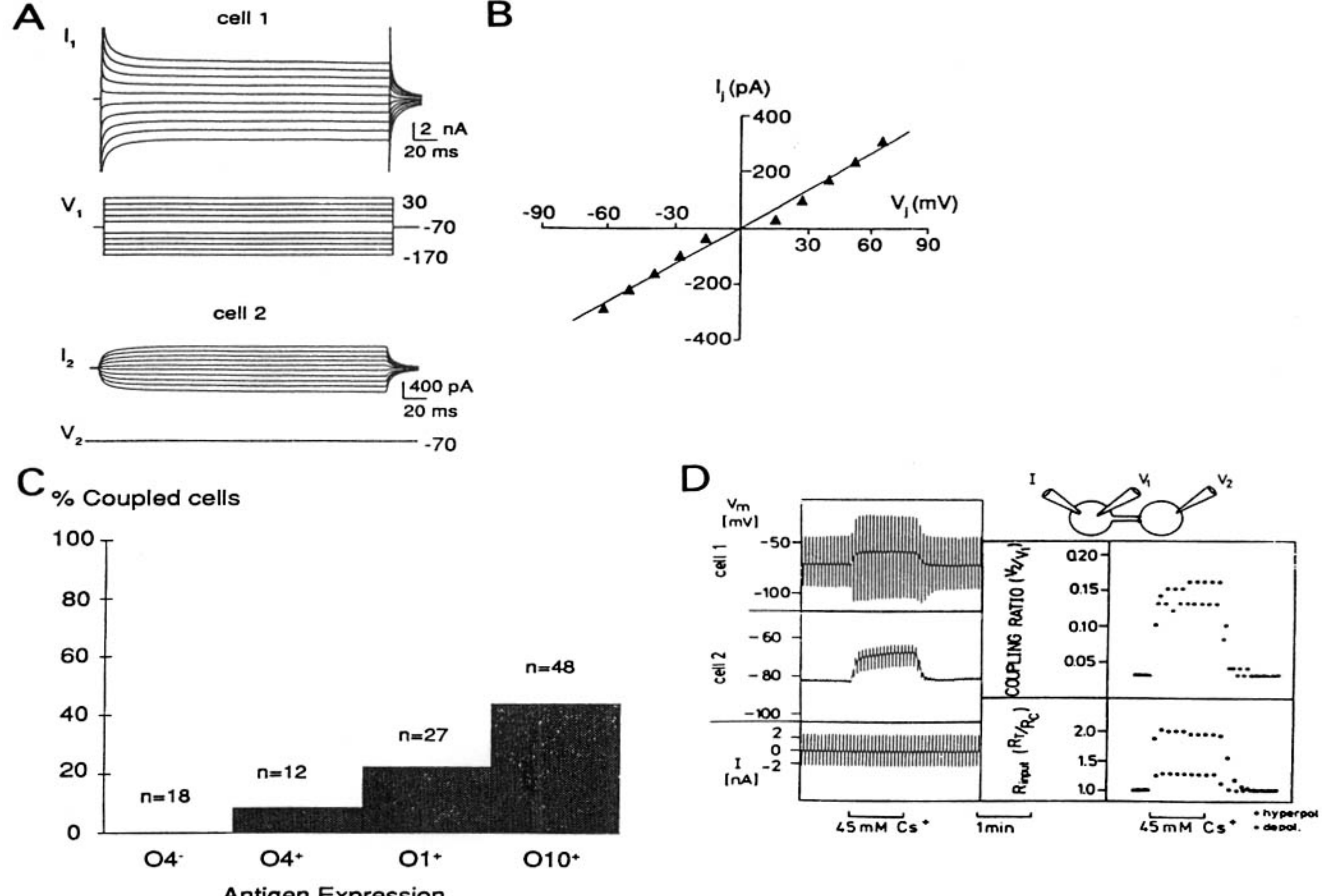


FIG. 19-8. Characteristics of dye and electrical coupling between cultured oligodendrocytes. (A) Two adjacent mature oligodendrocytes are voltage-clamped using the double whole-cell patch clamp configuration. In one cell of the pair the voltage is stepped between −170 and +30 mV (V_1). The second cell is kept at −70 mV (V_2). The currents resulting from these voltage steps are shown for both cells. (B) Based on the measurements made in A, the current flowing across the gap junctions between these cells, I_j, can be calculated and is plotted as a function of transjunctional voltage (V_j). The gap junctional conductance estimated from this relationship is 4.2 nS. (C) Coupling between oligodendrocytes increases with maturation as defined by the expression of stage specific antigens; immature oligodendrocytes express O4 and only mature oligodendrocytes express the O10 antigen. [From Blankenfeld et al. (1993), with permission.] (D) Cesium (Cs^+) increases coupling ratio in oligodendrocytes. Coupling is monitored by injecting current pulses (I) into one oligodendrocyte while recording membrane potential (V_m) in the same cell (cell 1) and in an adjacent oligodendrocyte (cell 2). Application of Cs^+ (substituted for Na^+) reversibly increases coupling ratio. Cs^+ blocks resting K^+ conductance (note increase in input resistance) and is believed to increase coupling ratio by increasing the effective length constant of the glial membrane without any direct effect on junctional resistance. Solid circles are measurements from depolarizing pulses and open circles are measurements from hyperpolarizing pulses. [From Kettenmann and Ransom (1988), with permission.]

which increases coupling ratio three- to fivefold), coupling is seen in about 80% of the contiguous cell pairs tested (Ransom and Kettenmann, 1990). In rabbit retina, dye is able to flow more freely from astrocytes into oligodendrocytes than from oligodendrocytes into astrocytes (Robinson et al., 1993). This interesting observation of unidirectional coupling, or "chemical rectification," may be the consequence of a gap junction composed of two hemiconnexons, each composed of a different connexin, namely, connexin43 in the astrocyte and connexin32 in the oligodendrocyte. It is not clear if unidirectional coupling is a general feature of astrocyte-oligodendrocyte junctions, but to the extent that it is, it suggests the possibility of a hierarchical arrangement for the flow of some metabolic signals (Robinson et al., 1993).

Other Glial Cells. As mentioned above, Schwann cells exhibit coupling which disappears when they begin to make myelin (Konishi, 1990). These junctions are probably mediated by connexin32 (Bergoffen et al., 1993), which continues to be expressed in Schwann cells in mature myelinated rat sciatic nerve, but is confined to the paranodal regions and

Schmidt-Lanterman incisures. This pattern suggested that gap junctions may connect the paranodal folds of myelinating Schwann cells, perhaps facilitating ion and small molecule movements to and from the tight, periaxonal space. The X-linked form of the hereditary neuropathy known as Charcot-Marie-Tooth disease, is associated with mutations in the connexin32 gene (Bergoffen et al., 1993). These observations indicate that gap junction protein expressed in Schwann cells plays a critical role in peripheral nerve integrity and function.

Ependymal cells, specialized glia which line the ventricles, have gap junctions and exhibit physiological properties that are similar to other glial cells (Connors and Ransom, 1987; Jarvis and Andrew, 1988; see also Ransom and Carlini, 1986). Turtle ependymal cells are extensively coupled to one another and are uncoupled by exposure to elevated CO_2 (Connors and Ransom, 1987). Similar observations have been made for ependymal cells in the rat third ventricle, but these cells seemed resistant to uncoupling by intracellular acidification (Jarvis and Andrew, 1988). Gap junctions in ependyma are constructed of connexin26 and connexin43 (Dermeitzel et al., 1989).

As discussed above, nonmammalian Müller cells, but not mammalian Müller cells, exhibit gap-junctional communication. Müller cells in the salamander retina show strong coupling to one another with coupling ratios as high as 0.2 at distances of 200 μm. Theoretical calculations indicate that electrical coupling in this system may significantly contribute to lateral movement of accumulated K^+ within the retina (Mobbs et al., 1988).

Bergmann glial cells of the cerebellum, are coupled to each other *in situ* (H. Kettenmann, personal communication). Recently, glial cells of the intermediate lobe of the pituitary, called stellate glia, were found to be strongly coupled to one another (Mudrick-Donnon et al., 1993). These cells also receive synaptic-like contacts from neurons, and the fascinating observation was made that they exhibit stimulus-evoked synaptic-like potentials mediated by the inhibitory transmitter GABA.

Functions of Gap Junctions

Several functions have been proposed for gap junctions based on their physiological properties (Loewenstein, 1981; Caveney, 1985; Bennett et al., 1991). They may serve to coordinate the electrical and metabolic activities of cell populations, act to amplify the consequences of signal transduction, control intrinsic proliferative capacity and help to orchestrate the complex events of embryonic morphogenesis. In some tissues, but not in others, the practical significance of intercellular coupling is clear. For example, the coordinated contraction of the heart, critical for its function as a pump, is mediated by rapid transfer of voltage signals via gap junctions between individual heart cells (Loewenstein, 1981). We do not, however, have detailed information about how coupling contributes to the functions of glial cells, but plausible hypotheses may be formulated that are variations on the general themes listed above. The robust and selective expression of coupling in glial cells suggests that intercellular communication is of major importance for the functioning of these cells within the brain.

The pattern and strength of coupling among different types of glial cells should be born in mind as possible functions of these cells are discussed. Available evidence indicates that astrocytes are strongly coupled to one another, while coupling between oligodendrocytes is weak, and heterologous coupling between astrocytes and oligodendrocytes is weaker still (Figure 19-9). Astrocytes, therefore, are likely to participate more vigorously than oligodendrocytes in most of the functions related to intercellular coupling. The geometry of coupling among cells could be important in determining function. For example, astrocytes in certain areas of the brain might be cou-

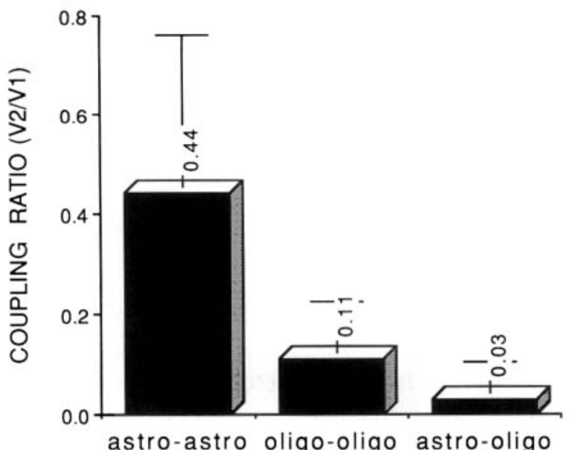


FIG. 19-9. Comparison of homogolous and heterogolous glial coupling. The mean coupling ratios are shown for oligodendrocyte pairs, astrocyte pairs, and astrocyte-oligodendrocyte pairs. All the cell pairs are within 100 μm of each other. Cell pairs which failed to show coupling have been excluded, since coupling ratio cannot be calculated under this circumstance. [From Ransom and Kettenmann (1990), with permission.]

pled in nonrandom patterns conferring a directional preference on intercellular communication. Thus far, such patterns have not been detected *in situ* (Gutnick et al., 1981; Binmöller and Müller, 1992), but this remains an open question. Propagating waves of Ca^{2+} increase in astrocytes (see below), stimulated by glutamate, seem to move in a nonuniform fashion through astrocytic monolayers, suggesting that this signal may follow specific pathways through coupled glial aggregates (Finkbeiner, 1993). The significance of coupling between astrocytes and oligodendrocytes remains obscure. Astrocytic processes which project to nodes of Ranvier, called *perinodal astrocytes*, are frequently connected by gap junctions to the terminal paranodal loops of nearby myelinating oligodendrocytes (Black and Waxman, 1988). This specialized arrangement could mediate an axon-specific type of K^+ spatial buffering (see below). To move beyond mere speculation, however, more information is needed about the relative strengths and patterns of intercellular communication among glial cells *in vivo*.

Potassium Ion Spatial Buffering and Extracellular Potential Generation. Astrocytes exhibit strong electrical coupling to one another, which may assist K^+ spatial buffering and the generation of extracellular potentials. The K^+ spatial buffer hypothesis states that glial cells participate in moving extracellular K^+ from areas where it accumulates secondary to neural activity, to distant areas where the K^+ is not elevated (Orkand et al., 1966; Chapter 37, this volume). This hypothesis remains attractive, but its quantitative significance has been difficult to establish (Ransom and Carlini, 1986; Sykova, 1983; Karwoski et al., 1989; Chapter 37, this volume). Strong astrocytic coupling would extend the distance over which this K^+ distribution system could operate and, perhaps, increase its efficiency (Orkand, 1986; Mobbs et al., 1988). Some experimental evidence supports this notion. In the isolated frog spinal cord, massive increases in P_{CO_2}, which presumably uncoupled glial cells due to intracellular acidification (but see Kettenmann et al., 1990), slowed the clearance of neural activity-induced increases in K^+ (Sykova et al., 1988).

Another mechanism whereby glial cells may participate in buffering the extent of K^+ accumulation occurring with neural activity, is by direct uptake and sequestration of K^+ (Coles and Tsacopoulos, 1979; Ransom and Carlini, 1986; Ballanyi et al., 1987). This process might also benefit from glial coupling because the coupled glial aggregate would have a larger intracellular volume to buffer against changes in intracellular K^+ concentration that could occur with K^+ uptake. This would be desirable because the K^+ uptake system (e.g., passive KCl uptake and/or KCl cotransport and/or Na^+-K^+ ATPase; see Ransom and Carlini, 1987; Chapter 18, this volume) would tend to function better against a lower K^+ concentration gradient.

It is well established that glial cells participate in the production of extracellular field potentials seen with neural activity (Ransom and Carlini, 1986; Ransom, 1974; Somjen, 1970). This has been most elegantly studied in the mudpuppy optic nerve, where the consequences of direct glial depolarization on surface potential can be quantitatively analyzed (Cohen, 1970). It is apparent from this work that interglial coupling is critical for determining the distribution of extracellular field potentials (Cohen, 1970). Glial-generated field potentials may influence neural integration, but the magnitude of such effects is unknown (see Somjen, 1973).

Intracellular Metabolic Homeostasis. The best understood role of gap-junctional communication is to provide for synchonization and propagation of electrical activity in certain excitable cells, namely, heart and smooth muscle cells, and some neurons (Loewenstein, 1981). In nonexcitable cells, gap junctions may be more important for metabolic coordination within the coupled aggregate. In the case of cells that are strongly coupled, uniform distributions of ions and small molecules are ensured, and cellular variations in the concentrations of junction-permeant molecules will be buffered. For example, frog skin cells exhibit similar Na^+ concentrations when the cells are coupled, but the concentrations diverge when the cells are uncoupled (Rick et al., 1981). Gap junctions, therefore, can cause a group of individual cells to function almost as one in terms of metabolism and membrane potential (Figure 19-10A).

The capacity for small molecules to diffuse freely between coupled glial cells has several theoretical advantages. Glial cells have high affinity uptake systems for a number of neurotransmitters (Chapter 48, this volume). Under normal conditions, transmitters are released focally at synaptic sites and transported across immediately adjacent glial membranes, which constitute only a small fraction of a given glial cell's total surface area. The rate of transmitter uptake will depend, among other things, on the transmembrane gradient for this molecule. Strong glial coupling, by providing a larger effective intracellular volume, would prevent large changes in the intracellular concentration of the transmitter from occurring with uptake, maximizing the efficiency of this process.

Conversely, transmitter molecules can also be re-

leased from glial cells, although the functional significance of this is poorly understood (Chapter 48, this volume). Release from glial cells is nonvesicular and will depend, in part, on the intracellular concentration of the transmitter. Coupling among glial cells would ensure relatively stable intracellular levels of various transmitter molecules, and ensure uniformity of the release process.

Genetically aberrant cells incapable of manufacturing certain metabolites can survive by receiving the metabolite in short supply via gap junctions with healthy neighboring cells (Loewenstein et al., 1981). At present, this remains only a speculative possibility for glial cells.

Signal Transfer. Recently a novel form of cellular response has been identified that consists of a propagating wave of cytoplasmic Ca^{2+} increase (Finkbeiner, 1993; Chapter 17, this volume). Waves of cytoplasmic Ca^{2+} increase are readily elicited in astrocytes by glutamate application. The waves move through individual cells at a constant velocity and follow a somewhat curvilinear course. Functional gap junctions are necessary for the intercellular propagation of glutamate-induced Ca^{2+} waves (Finkbeiner, 1993). Current evidence suggests that intercellular propagation of Ca^{2+} waves is mediated by the movement of inositol 1,4,5-trisphosphate (IP_3) through gap junctions (Boitano et al., 1992); Ca^{2+} is then released from intracellular stores by the traveling wave of IP_3. The functional importance of this intriguing long-range signaling system in astrocytes is unknown, but it depends on gap-junctional communication.

Another functional consequence of intercellular coupling is divergence of signal transmission (Figure 19-10B). Consider the case of a cell stimulated to produce an intracellular second messenger molecule (e.g., cAMP, IP_3, Ca^{2+}) by binding a specific ligand. If the cell is not connected to its neighbors by gap junctions, it will act in isolation. But if the cell is

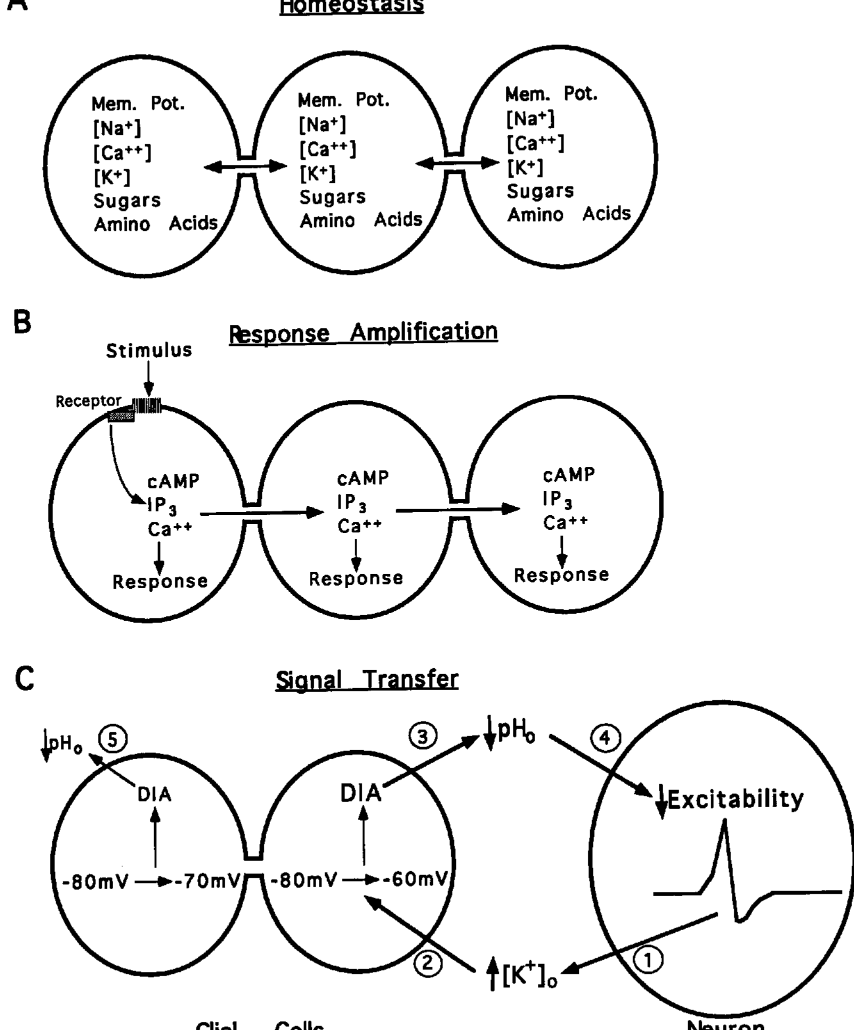


FIG. 19-10. Schematic illustration of some of the possible functions of intercellular communication. (A) In strongly coupled cell aggregates, the intracellular concentrations of ions and molecules less than ~1,000 kD in size (including amino acids, sugars, and second-messenger molecules) will tend to be similar due to free exchange across gap junctions. This will cease to exist if "gating" conditions favor closure of gap channels; pathological circumstances such as anoxia may lead to rapid uncoupling because of increases in intracellular Ca^{2+} and H^+. (B) Coupling can amplify the physiological consequences of a chemical stimulus (i.e., hormone, neurotransmitter, etc.) acting on a single cell. Such stimuli often cause the production of second messenger molecules (i.e., Ca^{2+}, IP_3, cAMP) that can move easily into adjacent cells via gap junctions; the coupled cells are then recruited to respond. (C) Schematic illustration of a hypothesized neuronal-glial interaction mediated by pH_0 (Ransom, 1992). Active neurons cause an increase in $[K^+]_0$ (*1*), which causes depolarization (*2*) leading to depolarization-induced-alkalinization (*DIA*). The glial DIA causes an acid shift in pH_0 (*3*) that decreases the excitability of nearby neurons (*4*). Distant glial cells that are coupled by gap junctions to glia in the immediate vicinity of the active neurons, also experience the depolarization "signal," but less strongly. These distal cells then weakly acidify their environment (*5*), perhaps producing reduced excitability in the neurons that surround the actual area of elevated $[K^+]_0$ (Ransom, 1992). [Figure C modified from Ransom (1992), with permission.]

widely coupled to other cells, the diffusable, junction-permeable second messenger (Saez et al., 1989) has the potential of activating many cells (Loewenstein, 1981).

Kuffler and his colleagues speculated that K^+ accumulating in brain extracellular space with neural activity might act as a signal to glial cells because these cells responded to K^+ with graded membrane depolarization (Orkand et al., 1966; Kuffler and Nicholls, 1967; Chapter 31, this volume). Recent observations suggest that this local "signal" may cause an extracellular acid shift that is mediated by depolarization of glial cells (Ransom, 1992). The glial-mediated acid shift, in turn, may modulate ongoing neural activity. This hypothesized neuronal-glial interaction can potentially be amplified by glial coupling as shown in Figure 19-10C. The presence of strong coupling would be predicted to produce a larger area of extracellular acidification and thereby a larger area of neural modulation (Ransom, 1992).

Proliferative Activity and Development. The role of gap-junctional communication during development has been discussed at length (e.g., Caveney, 1985). Embryos exhibit widespread gap-junctional communication, which may be important in establishing developmental fields critical for morphogenesis. Until more is known about the molecules that direct early development, however, specifically if they are junction-permeable, this attractive hypothesis remains difficult to test. Developing rat cortical neurons are transiently coupled to each other in columnar groups, and it is hypothesized that this coupling may play a role in determining the functional organization of the cortex (Lo Turco and Kriegstein, 1991). Coupling between glial cells develops as these cells mature, and would not, therefore, play a role in early glial development. Conceivably, coupling might be related to the determination, and perhaps maintenance, of final cellular phenotype.

Considerable circumstantial evidence indicates that gap-junctional communication can act to restrict cell proliferation (Loewenstein, 1981). Most malignant cell populations show no intercellular coupling (Loewenstein, 1981; Klaunig and Ruch, 1990; Bennett, et al., 1981). The mechanism by which gap-junctional coupling might serve to suppress mitotic activity is presently unknown. Because the majority of primary brain tumors are glial in origin, the possibility that loss of gap junctions in these cells leads to neoplastic transformation is intriguing. The observation that C6 glioma cells fail to exhibit gap junctions is consistent with this idea (Naus et al., 1993), as is the observation that highly malignant human gliomas lack gap junctions (Tani et al., 1973). More work will be necessary to properly test the importance of intercellular communication as a regulator of proliferation in glia.

CONCLUSIONS

Gap junctions and the special form of intercellular communication which they mediate, have been extensively investigated in the last quarter century since their discovery. Our knowledge about gap junctions is impressive but incomplete. This is especially so in the brain, where these junctions are widely expressed in glial cells, but we lack conclusive insight about how they participate in brain function. The situation, however, should be viewed optimistically because gap-junctional communication among glial cells has been sufficiently well characterized that reasonable hypotheses about their function can now be formulated and tested. Research prospects in this area are also brighter due to the availability of important new information about the molecular biology of gap junctions, which places in our hands powerful tools for further experimental analysis.

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20 Electrophysiology of mammalian glial cells *in situ*

GEORGE G. SOMJEN

Experiments on invertebrate neuroglia and on glial cells in culture have yielded many exciting discoveries that radically changed our conception of the place of these cells in general biology. The newly found wealth of receptors and ion channels expressed in the membrane of glial cells in such systems is described elsewhere in this volume. In this chapter the focus is on the electrophysiological responses of neuroglia manifested *in situ* in the central nervous system (CNS) of mature mammals. In experiments with intact central nervous system tissue, the gap between the ideal and the practical is usually greater than it is in experiments on model systems such as cell cultures or invertebrate ganglia. Still, in spite of technical difficulties, our understanding of mammalian glial cells would be incomplete without observing them in their natural habitat, under as nearly physiological conditions as is technically feasible.

The scope of this chapter, then, is limited. It covers only electric responses of mammalian glial cells as seen either in intact CNS or in those isolated tissue preparations that have been freshly dissected from mature CNS with their cytoarchitecture more or less preserved. Inclusion of data from brain tissue slices seems justified in the light of the similarity of glial cell responses in such preparations to those in intact brain (Schwartzkroin and Prince, 1979; Casullo and Krnjević, 1987). The early literature on glial electrophysiology has been repeatedly surveyed (Kuffler and Nicholls, 1966; Kuffler, 1967; Somjen, 1975; Somjen, 1980; Somjen, 1987), and only the most relevant contributions are summarized here. Inevitably, this chapter overlaps to some degree with Chapters 11, 12, 18, 19, 31, and 47 and perhaps others in this volume. My goal is to avoid undue repetition yet present a cohesive essay that may be readable by itself. Therefore, areas in common with others will be included insofar as is necessary to present the main topic coherently.

MEMBRANE POTENTIAL RESPONSES OF GLIAL CELLS

Investigation of the cellular electrophysiology of the mammalian central nervous system began with the invention of intracellular recording, applied first to cat spinal cords (Brock et al., 1952), then to other regions. Nerve cells were hunted in blind searches with microcapillary electrodes in the depth of gray matter. Sometimes, instead of the sought-after neurons, cells were encountered that generated neither action potentials nor synaptic potentials (Phillips, 1956). Various authors called these cells "inexcitable," "silent," "unresponsive," *stumm* (=mute) and "idle." While careful not to commit themselves, most workers suspected that they were glia (e.g., Phillips, 1956; Sugaya et al., 1964; Krnjević and Schwartz, 1967; Glötzner and Grüsser, 1968). Eventually they were identified as such by *intra vitam* intracellular marking with dye followed by *post mortem* histology (Kelly et al., 1967; Grossman and Hampton, 1968; Kelly and Van Essen, 1974).

But as it turned out, glial cells are electrically not completely unresponsive after all. Sugaya et al. (1964) were the first to notice that the membrane potential of "idle" cells actually does change during neuronal excitation. They reported in 1964 that during seizure discharges and during spreading depression these cells underwent slow depolarization.

These early encounters with presumed glial cells were usually accidental. The first deliberately planned study of "idle" units in cerebral neocortex was probably by Karahashi and Goldring in 1966. They reported that repetitive stimulation of the cortical surface, or of an afferent pathway, caused a depolarizing shift of the membrane potential of these cells, which they believed could either be glia, or neuron dendrites, or nonspiking neurons. Single volleys, which did evoke sizable neuronal responses, had no effect on the "idle" cells. These observations were soon confirmed by others (Glötzner and Grüs-

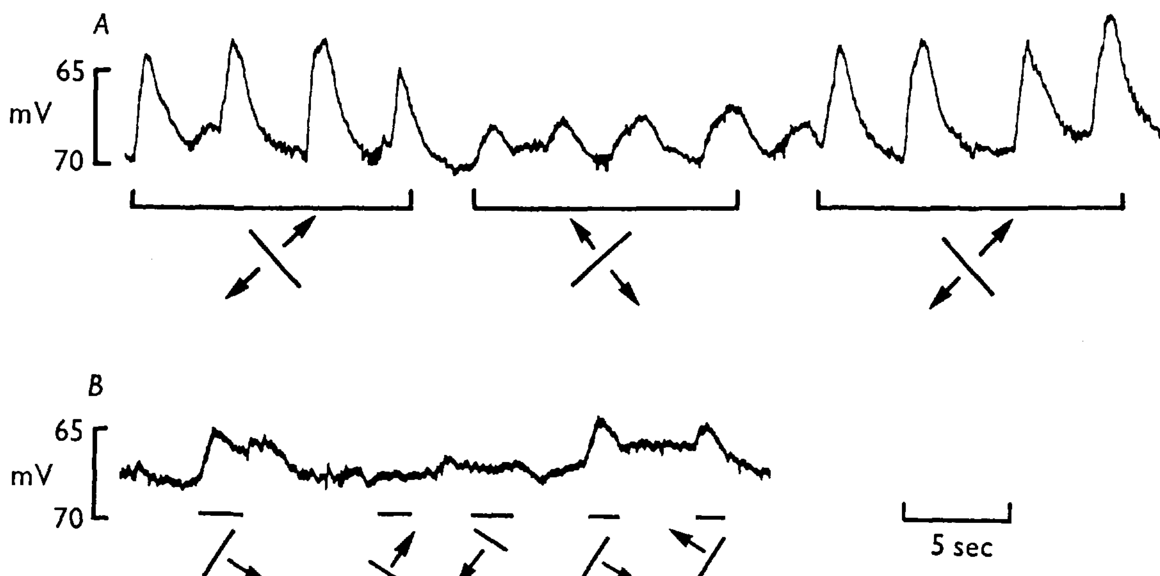


FIG. 20-1. Responses of cortical cells evoked by physiological (adequate) stimulation of sense organs. (A, B) Two glial cells, both in primary visual receiving area of the cerebral cortex of an anesthetized cat. The *insets* show the orientation and direction of the movement of the visual stimulus, which was a slit of light projected on a screen in the animal's visual field. The largest depolarizing responses are evoked by the stimulus oriented optimally for excitation of the neurons located in the same cortical column. [From Kelly and Van Essen (1974), with permission.]

ser, 1968; Grossman and Hampton, 1968; Sypert and Ward, 1971; Dichter et al., 1972; Ransom and Goldring, 1973b; Ransom, 1974). That not only electrical stimulation, but also more natural, adequate sensory stimulation, can evoke slow depolarizing responses was first reported by Kelly and Van Essen (1974). In these experiments the cells were marked by dye injected *in vivo* and their identity as glia was confirmed by histology *post mortem*. The now classical illustration of their responses is reproduced as Figure 20-1.

Slow depolarization during repetitive orthodromic stimulation was also recorded in spinal cord from "unresponsive" cells that were assumed to be glia

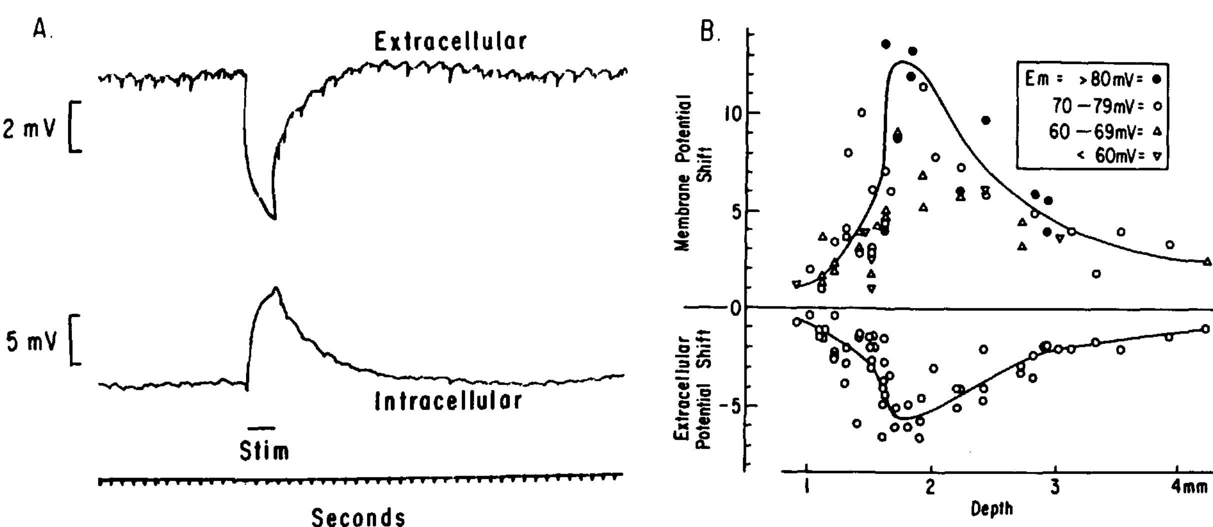
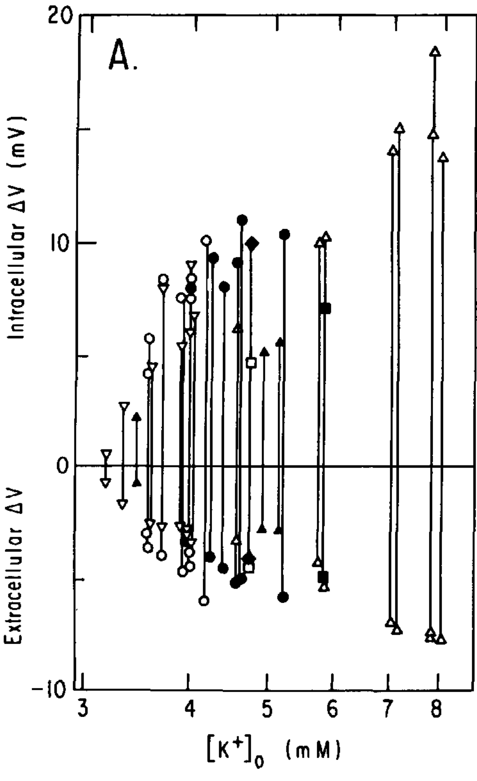


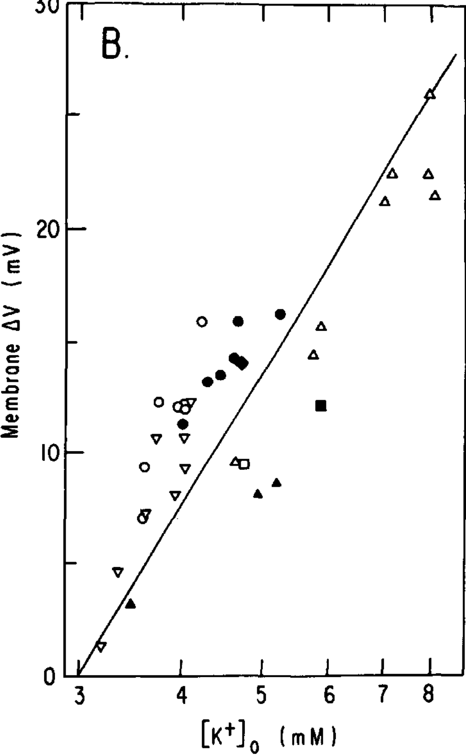
FIG. 20-2. Responses of glial cells and simultaneously recorded extracellular sustained potential shifts (ΔV_0) in spinal gray matter of cats. (A) Sample recordings made with twin microelectrodes fastened together so that the tips lay not more than 50 μm apart. Horizontal bar marks repetitive simulation of the dorsal root. (B) Amplitude of the responses evoked by identical trains of pulses in 7 cats. The abscissal scale shows depth from the dorsal surface of the spinal cord. The *inset* indicates the ranges of the resting potentials of the glial cells. [From Somjen (1970), with permission.]

(Somjen, 1969, 1970) (Figure 20-2). The depolarization of presumed glial cells differed from the synaptic potentials of neurons not only in the much slower time-course, but also in that it was not associated with a change of input resistance (Somjen, 1970). The same lack of change in input resistance was also noted for cortical glial cells (Sypert and Ward, 1971; Ransom and Goldring, 1973b). The slow depolarization of spinal and cortical glial cells resembled closely the responses recorded in glia of leech and amphibia by Kuffler and associates (Orkand et al., 1966; Kuffler and Nicholls, 1966; Kuffler, 1967). These authors found that depolarization of glial cells of cold-blooded animals was caused by the rise of extracellular potassium concentration ($[K^+]_0$), due to the release of K^+ ions from excited neurons. The membrane of these cells behaved as a "perfect K^+-electrode," and it had a constant ohmic resistance that showed no voltage-dependent changes (Orkand et al., 1966; Kuffler and Nicholls, 1966; Kuffler, 1967).

Confirming the similarity with leech and amphibia, a close correlation of glial membrane potential and $[K^+]_0$ was also evident in recordings from glial cells in mammalian neocortex, optic nerve, and spinal cord (Dennis and Gerschenfeld, 1969; Ransom and Goldring, 1973a, 1973b; Lothman and Somjen, 1975; Roitbak et al., 1981) (Figure 20-3). The relatively high negative resting membrane potential, and its dependence on $[K^+]_0$, refuted the earlier contention that glial cytoplasm is in effect the "extracellular space" surrounding the neurons of the CNS.

An early report on neocortical glia by Krnjević and Schwartz (1967) indicated that glial cells have high and stable membrane potential and high input resistance, without voltage-dependent responses, action potentials, or synaptic potentials. Nor did glutamate, 5-hydroxytryptamine (5-HT), or norepinephrine administered by microiontophoresis have an effect on the membrane potential. Potassium ions released from an iontophoretic source did cause depolarization, confirming that the membrane poten-





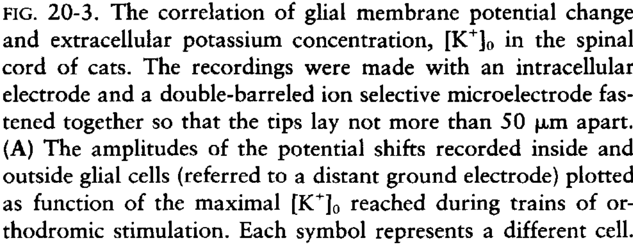


FIG. 20-3. The correlation of glial membrane potential change and extracellular potassium concentration, $[K^+]_0$ in the spinal cord of cats. The recordings were made with an intracellular electrode and a double-barreled ion selective microelectrode fastened together so that the tips lay not more than 50 μm apart. (A) The amplitudes of the potential shifts recorded inside and outside glial cells (referred to a distant ground electrode) plotted as function of the maximal $[K^+]_0$ reached during trains of orthodromic stimulation. Each symbol represents a different cell. Each cell was stimulated by several pulse trains of varying intensity and frequency applied to an afferent nerve. The vertical lines connect the simultaneously obtained data. (B) Transmembrane potential changes calculated as the sum of the extracellular and intracellular potential shifts, as function of maximal $[K^+]_0$ reached during stimulation. The line shows the Nernst function at 37.5°C, calculated assuming constancy of intracellular K^+ concentration. [From Lothman and Somjen (1975), with permission.]

tial was K^+-dependent. Large doses of γ-aminobutyric acid (GABA) caused depolarization of the unresponsive cells without changing input resistance, unlike neurons, which hyperpolarize under the influence of GABA with concomitant decrease of membrane resistance. Acetylcholine (ACh) had an action similar to GABA on some but not all "unresponsive" cells. The constancy of the input resistance was inconsistent with the opening (or closing) of ion channels, and Krnjević and Schwartz (1967) attributed the depolarization by GABA and ACh to electrogenic active uptake of these transmitter agents by the presumed glial cells.

Acknowledging conceptual precedence, Krnjević and Schwartz (1967) quoted an early essay by Lugaro (1907), who believed that one of the main functions of glia was to remove unwanted matter from cerebral interstitial spaces. This included elimination of chemical substances elaborated by nerve terminals that influence the dendrites of nerve cells. He wrote: "Protoplasmic extensions of glial cells which invariably penetrate to the vicinity of neural articulations serve to collect and to instantly break down any minimal residue of such chemical agents." With minor change of terminology, this quote could be from a review article datelined yesterday (see also Somjen, 1988).

The idea of electrogenic neurotransmitter transport by glia has recently received experimental support from Attwell's group (Brew and Attwell, 1987; Barbour et al., 1988; Sarantis and Attwell, 1990; Szatkowski et al., 1990). They found that exposure to glutamate induced a depolarizing inward current in retinal glial cells. Some, but not all, other types of neuroglia responded similarly (Schwartz and Tachibana, 1990; Wyllie et al., 1991). The current has been attributed to the influx of positively charged Na^+ ions in excess of the cotransported anionic transmitter molecules. It is activated by intracellular K^+ and inhibited by a rise of extracellular K^+ concentration ($[K^+]_0$). The uptake current is distinguished from receptor channel current by the absence of channel "noise," no change of membrane resistance, and no definable reversal potential.

THE CONTRIBUTION TO EXTRACELLULAR SUSTAINED POTENTIAL SHIFTS BY GLIAL CELLS

The origin of "slow" or "direct current," or "sustained" extracellular potential shifts, has puzzled electrophysiologists for some time. From the beginning, most investigators attributed the generation of sustained potentials either to the dendritic arbor of neurons or to glia (for review of the early literature, see Somjen, 1973). Karahashi and Goldring (1966) supplied the first experimental support for these speculations: they found that the depolarization of "idle" cells correlated with the slow negative cortical surface potentials. As mentioned earlier, at the time they too hesitated between identifying the cells as either nonspiking neuronal elements or glia. Others confirmed the basic observation (Glötzner and Grüsser, 1968; Castellucci and Goldring, 1970).

The mechanism by which glial tissue could generate extracellular potential shifts was suggested by Kuffler and associates (Kuffler and Nicholls, 1966; Kuffler, 1967; Cohen, 1970), in large part based on observations on optic nerve of amphibia. In this tissue glial cells appeared linked by gap junctions, forming a quasi-syncytial electrotonic net (see Chapter 19, this volume). Unequal depolarization of the cells so linked could generate a current flowing through and around the electrotonic net, and this current could give rise to a shift of extracellular potential that could be recorded by extracellular electrodes. These ideas were expressed in a formal model by Joyner and Somjen (1973). The model defined the relative magnitude and distribution of potential shifts around an electrotonic net as functions of the relative magnitudes of the resistances of the intercellular junctions, of the bounding membrane, and of the interstitial environment (Figures 20-4 and 20-5).

In cat spinal cord, repetitive stimulation of peripheral or dorsal root afferent fibers evokes unusually large extracellular sustained potential shifts (ΔV_0), always of negative polarity throughout the extension of the gray matter (Somjen, 1969, 1970) (Figure 20-2). The large amplitude and reproducibility of these signals facilitated their analysis (Somjen, 1970). Neither magnitude nor polarity of the sustained ΔV_0 correlated with the excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs) recorded from neurons. By contrast, there was a close correspondence between the depolarization of presumed glial cells and the sustained negative ΔV_0 recorded simultaneously in their immediate vicinity (Figure 20-6). This correlation held well when the recording microelectrode pair was advanced in small steps through the tissue, and a constant stimulus train was repeated at every station (Figure 20-2); and also when the recordings were made from inside and outside of a single cell while the stimulus intensity or frequency were varied (Figure 20-3). Moreover, the regression of ΔV_0 on glial depolarization remained unchanged even when both variables were reduced in amplitude by depressant drug treatment (Strittmatter and Somjen, 1973).

The introduction of ion-selective microelectrodes made the simultaneous recording of interstitial potassium concentration ($[K^+]_0$) and ΔV_0 possible.

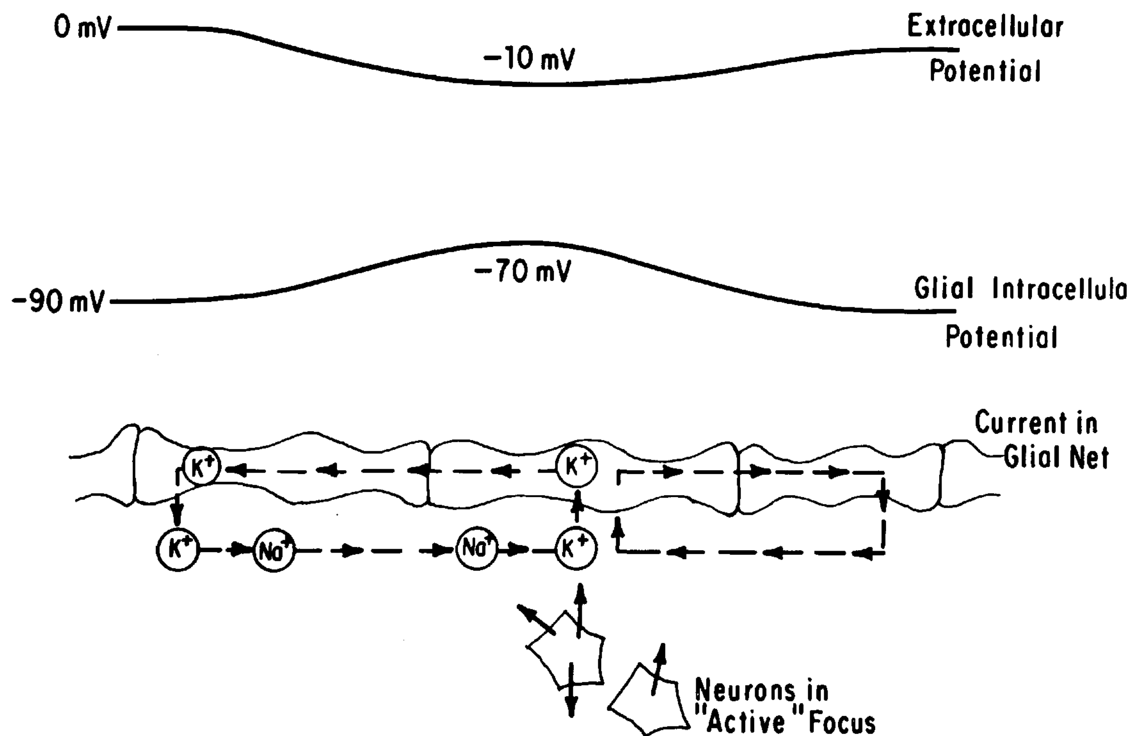


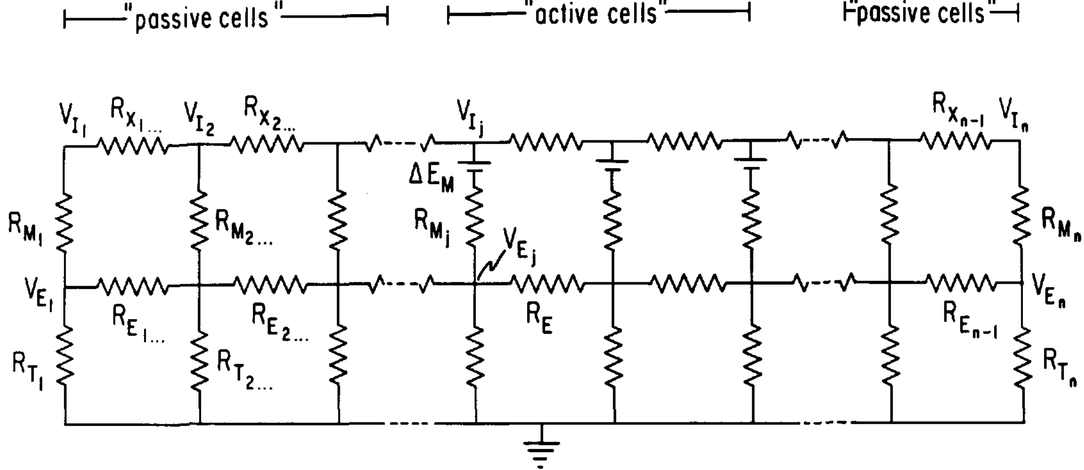
FIG. 20-4. Simplified model of the generation of extracellular ΔV_0 by the depolarization of glial cells. Near the center of the row of glial cells neurons are assumed to have released an excess of K^+ ions into the interstitial fluid. This causes depolarization of the adjacent glial cells, resulting in current flow through the gap junctions connecting the row of glial cells. The extracellular ΔV_0 corresponds to the "return" path of the current loop. Compare these hypothetical voltage profiles with the average potential profiles shown in Figure 20-2B. [From Somjen (1981), with permission.]

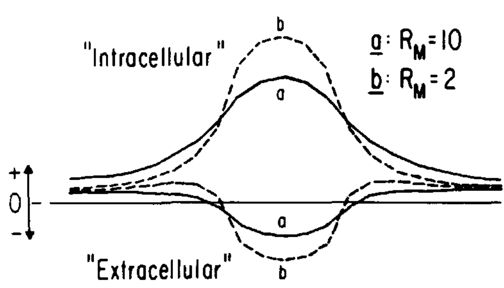
Combining such double-barreled ion selective electrodes with an intracellular electrode, the three-way correlation of sustained ΔV_0, $\Delta[K^+]_0$ and glial depolarization were confirmed. Moreover, the slope of the linear function linking the measured values of $[K^+]_0$ and glial membrane potential equaled the slope of the Nernst function calculated for a reasonably expected ratio of $[K^+]_0$:$[K^+]_i$ in mammalian glial cells (Lothman and Somjen, 1975) (Figure 20-3). This observation supported two propositions: (1) that the level of $[K^+]_0$ is the dominating influence on glial membrane potential; and (2) that the main generator of sustained ΔV_0 in cat spinal cord is the depolarization of glial cells. Indirectly, this set of observations also supported the idea that glial cells are linked by electrically patent junctions.

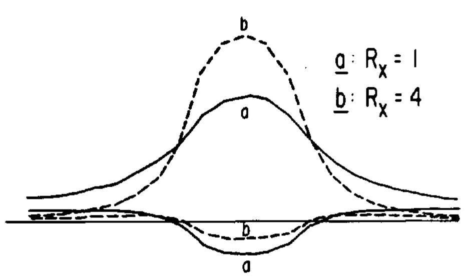
A close correlation between ΔV_0 and $\Delta[K^+]_0$ (more precisely, the logarithm of $\Delta[K^+]_0$) is a necessary but not a sufficient criterium for attributing a voltage shift to glial generation. As in spinal cord so also in cat's neocortex, the two, ΔV_0 and $\Delta[K^+]_0$, appeared correlated, but in neocortex sustained ΔV_0 responses were smaller for a given $\Delta[K^+]_0$ than in spinal cord (Lothman et al., 1975; Cordingley and Somjen, 1978; Somjen, 1978) (Figure 20-7). In other studies the depolarizing responses of glial cells also showed a relationship either to surface potentials or to interstitial ΔV_0 in many but not in all circumstances (Karahashi and Goldring, 1966; Castellucci and Goldring, 1970; Krnjević and Morris, 1972; Krnjević and Morris, 1975; Lothman et al., 1975; Cordingley and Somjen, 1978; Heinemann et al., 1979; Roitbak et al., 1981; Roitbak et al., 1987).

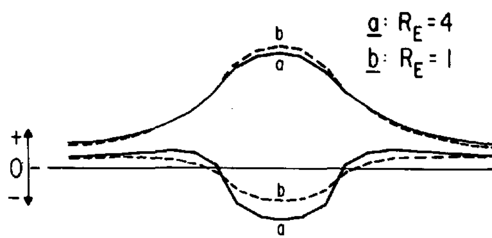
Both the smaller amplitude of ΔV_0 and its less secure correlation with $\Delta[K^+]_0$ and glial membrane potential indicate that the contribution of glia to the sustained ΔV_0 is less in cortex than in spinal cord (Figure 20-7). This could, in principle, be explained in at least three ways (Joyner and Somjen, 1973; Somjen, 1973): (1) a smaller relative size of the glial "compartment"; (2) lesser degree of electrotonic coupling, that is, fewer gap junctions or higher junctional resistances; (the higher input resistance of cortical compared to spinal glial cells (Somjen, 1975) is compatible with this explanation); and (3) lesser depolarization of cortical glia for a given $\Delta[K^+]_0$ (i.e., lesser selectivity of membrane K^+ permeability, e.g., relatively greater g_{Na^+} or "leak" conductance).

It has sometimes been argued that the low









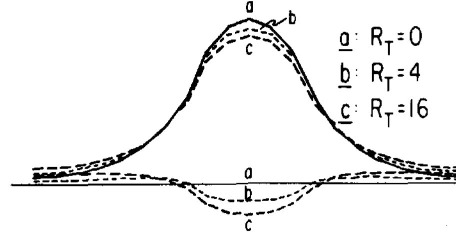


FIG. 20-5. Formal model and computer simulation of the network proposed in Figure 20-4. The upper diagram is the electric equivalent circuit; the lower four diagrams (A-D) are the numerical solutions, each for different relative values of the hypothetical resistances. "Active cells" are those assumed to be depolarized by excess $[K^+]_0$; "passive cells" those that are surrounded by normal $[K^+]_0$ but depolarized by current injected from the "active" cells. V_I, intracellular potential of glial cells; V_E, extracellular potential; R_X, coupling resistance of gap junctions connecting glial cells; R_M, membrane resistances; R_E, interstitial resistance; R_T, tissue resistance to ground; ΔE_M, "batteries" representing the depolarization caused by excess $[K^+]_0$, A-D show voltage profiles computed for varying relative magnitudes of the resistances. Compare these profiles with Figure 20-2B and Figure 20-4. [From Joyner and Somjen (1973), with permission.]

membrane resistance of glial cells precludes generation of sizeable extracellular voltages. Appropriate calculations show, however, that lowering membrane resistance (R_m) in a quasi-syncytial net does not reduce ΔV_0. The lowering of R_m does limit how far ΔV_0 spreads away from a depolarized zone, but it will actually increase the relative size of ΔV_0 within the depolarized zone. As a result, with low R_m, ΔV_0 will map in space the $\Delta[K^+]_0$ more accurately. If R_m is high, ΔV_0 spreads far outside the depolarized zone; if R_m is low,

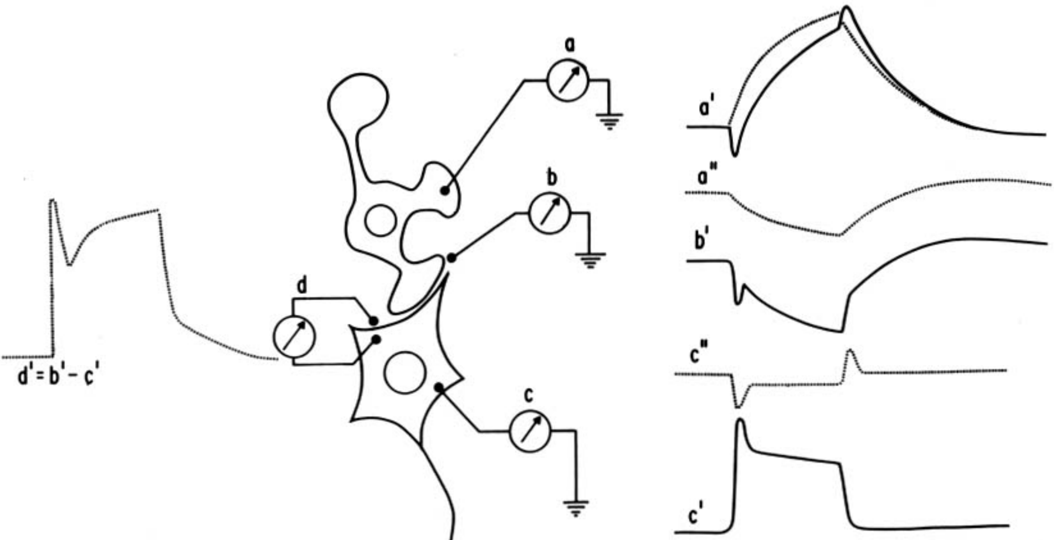


FIG. 20-6. Schematic illustration of responses of spinal cord neurons and glial cells evoked by orthodromic train stimulation, and their contribution to extracellular potential shifts, *a*, *b*, and *c* show conditions of the recordings from a glial cell, from the interstitial space, and a neuron, all referred to ground. *a'*, *b'* and *c'* show "smoothed" typical recordings from these three positions. *a"* and *c"* represent the contributions made by glia and neurons which sum to produce the extracellular ΔV_0 shown as *b'*. *d'* is the algebraic sum of neuronal intra- and extracellular potential changes, that is, the actual transmembrane potential change (EPSP) of a neuron. [From Somjen (1970), with permission.]

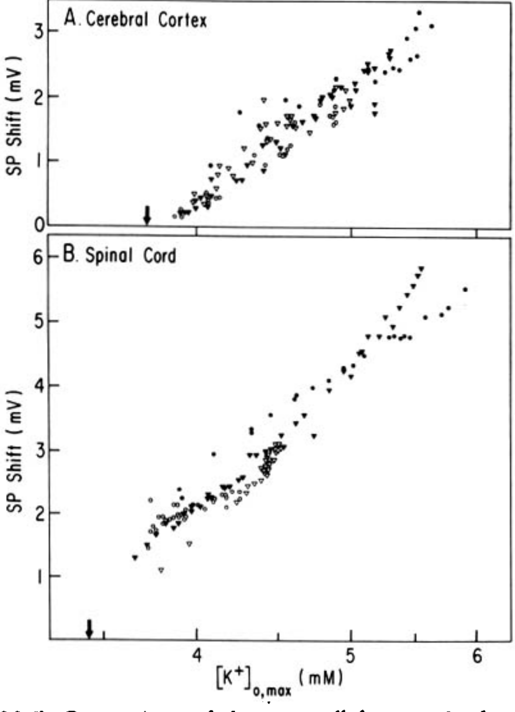


FIG. 20-7. Comparison of the extracellular sustained potential (SP or ΔV_0) shifts corresponding to varying $[K^+]_0$ levels reached during afferent train simulation in cerebral cortex and in spinal cord. Each point is the amplitude of one ΔV_0. The recordings are from a fixed site, in primary somatosensory cortex for *part A*, in the spinal dorsal horn for *part B*; responses in cortex evoked by stimulation of VPL nucleus of the thalamus; in the spinal cord by stimulation of the dorsal root. Different symbols represent series of responses obtained by varying different stimulus parameters (frequency, intensity, train duration). Note that $[K^+]_0$ is scaled logarithmically. [Experiment by G. E. Cordingley; from Somjen (1978), with permission.]

ΔV_0 remains confined to the depolarized region (Joyner and Somjen, 1973; Somjen, 1987) (Figure 20-5).

I have in this section considered the glial generation of ΔV_0 *per se*, disregarding the presumed role of glia in the "spatial buffering" of $\Delta[K^+]_0$. The role of neuroglia in regulating $[K^+]_0$ is discussed in detail in Chapter 47.

WHERE THE GLIAL CONTRIBUTION TO SUSTAINED EXTRACELLULAR POTENTIAL SHIFTS SEEMS NEGLIGIBLE

Casullo and Krnjević (1987) have recorded from glial cells in rat hippocampus. These cells could be made to depolarize by repetitive orthodromic volleys, just like their neocortical and spinal counterparts. But when the electrode was withdrawn into the interstitium, the same stimulus train usually did not evoke a detectable sustained ΔV_0. These cells did not seem to generate an extracellular current of sufficient magnitude to be detectable as a ΔV_0 by the usual recording equipment.

For entirely different reasons we have come to a similar negative conclusion concerning glia in the hippocampal formation (Somjen et al., 1985; Somjen and Giacchino, 1985; Somjen, 1993). Among the cell somata of the dentate gyrus (i.e., stratum granulosum) during repetitive orthodromic volleys, $[K^+]_0$ begins to increase at stimulus intensities that do not

evoke a sustained ΔV_0. With stronger stimulation negative ΔV_0's appear and they grow as does $\Delta[K^+]_0$, but the correlation is far less perfect than in neocortex or spinal cord. Among the dendrites of the dentate gyrus (stratum moleculare) there is no correlation between these two variables at all. In the lower range of stimulation the sustained ΔV_0 becomes increasingly negative, but then, above a certain level, it becomes smaller with increasing stimulation even though $\Delta[K^+]_0$ continues to grow. Once the stimulus trains become strong enough to provoke a seizure, in stratum moleculare a positive ΔV_0 replaces the negative ΔV_0, while $\Delta[K^+]_0$ increases all the while monotonically (Somjen and Giacchino, 1985). Current source density analysis revealed that the ΔV_0 associated with seizures in CA1 of hippocampus as well as in the dentate gyrus has a sink in the layer of cell bodies, and source among apical dendritic arbors (Wadman et al., 1992). Both, the lack of correlation between ΔV_0 and $\Delta[K^+]_0$ and the distribution of current sources and sinks, suggested that in this case the contribution of neuroglia to the extracellular sustained potential shift was negligible compared to that of neurons.

Why are hippocampal neuroglia incapable of generating sustained ΔV_0? To properly answer this question we would need to know the relative size of the glial compartment, and the density and conductance of gap junctions between glial cells. This information is currently not yet available.

PATCH-CLAMP RECORDING OF WHOLE-CELL CURRENTS FROM GLIAL CELLS

The method of tight-seal patch-clamp recording made it possible to clamp the membrane potential of whole cells as well as excised membrane patches (Hamill et al., 1981). Patch-clamp electrodes have successfully been applied to glial cells in tissue slices (Czéh et al., 1992b; Barres, 1991a; Berger et al., 1991, 1992a, 1992b; Mudrick-Donnon et al., 1992; Steinhäuser et al., 1992, 1993; Müller et al., 1993).

A pressing question of current glial research is the expression in glia *in situ* in mature CNS of the numerous membrane responses that have been discovered in glial cells in isolation and in cell cultures (Walz and MacVicar, 1988; Barres et al., 1990, 1991a, 1991b; Ransom and Sontheimer, 1992). It appears that certain of the common transmitter substances do elicit membrane ion currents in some glial cells (MacVicar et al., 1989; Berger et al., 1992b; Müller et al., 1992, 1993; Jabs et al., 1993).

In experiments not yet published in full, G. Czéh (1991, 1992a, 1992b, 1994) used patch-clamping to record from neurons and glial cells in CA1 region of rat hippocampal slices during electrically provoked seizure discharges, spreading depression (SD), and hypoxic spreading depression-like depolarization. An extracellular electrode placed in st. pyramidale very near to the patch clamp pipette served two purposes: it monitored the behavior of the surrounding neuron population; and it was connected to the "reference" input of the amplifier. Use of a reference in the tissue instead of a distant ground in the bath insured clamping of the true membrane voltage even when the extracellular potential was changing. The "diagnosis" of glial cell was made by the usual criteria of high, stable resting membrane potential and absence of synaptic or action potentials in current clamp configuration. The membrane potential of neurons and glial cells was clamped at a somewhat more negative level than the resting potential, which was determined earlier under current clamp. Membrane currents were explored at intervals by ramp voltage commands, which started with a brief hyperpolarizing step, and then depolarized the membrane along a linear trajectory by 100 mV over 200 ms. From the trajectory of the current registered during the depolarizing ramp, current-voltage (I-V) plots were then derived.

I-V plots of glial cells always yielded a straight line, indicating the absence of voltage dependent conductance change, that is, "ohmic" behavior (Figure 20-8). When the extracellular electrode registered an electrographic seizure, spreading depression, or hypoxic spreading depression-like depolarization, the holding current of the patch electrode increased, consistent with a tendency of the glial membrane to depolarize. At the same time the I-V plot derived from depolarizing ramp commands was displaced to the right, indicating depolarization, but it remained a straight line (Figure 20-8). No indication of rectification or other voltage dependent change or conductance was detected. The slope of the I-V function did usually change. The glial membrane conductance increased by 5 to 12% during electrographic seizures and by 10 to 21% during hypoxic spreading depression-like depolarization. These conductance increases were much less than those seen in neurons under similar conditions (Czéh et al., 1994). Also unlike neurons, the change in conductance of glial cells was not precisely correlated with the increase of the holding current or the shift of the reversal potential (i.e., zero current potential). In the example of Figure 20-8, the conductance is actually decreased at the moment of maximal depolarization as signaled by the increase of holding current (Czéh et al., 1991, 1992a, 1992b, 1994).

These experiments confirmed under the more rig-

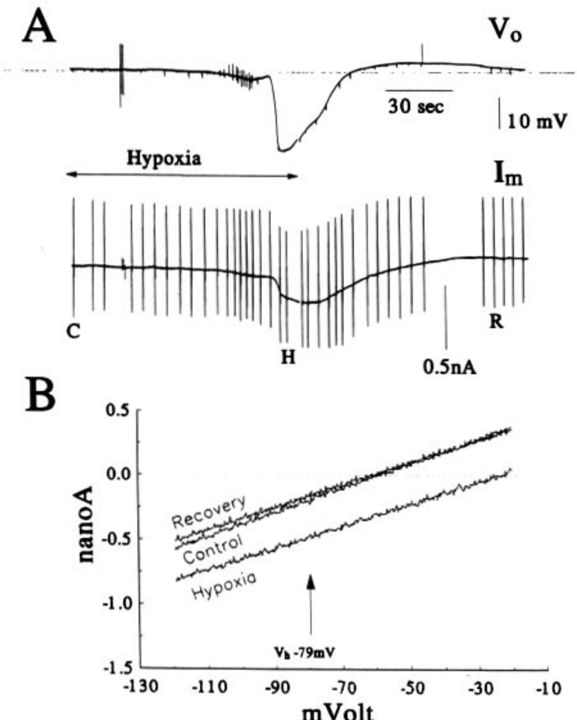


FIG. 20-8. Voltage-clamp recording from a presumed glial cell in CA1 zone of a rat hippocampal tissue slice. (A) Extracellular potential (V_0) and glial membrane holding current (I_m). The membrane potential was clamped at −79 mV. The horizontal *arrow* marked *Hypoxia* indicates the period during which N_2 replaced O_2 (with 5% CO_2) in the gas phase of the tissue chamber. The vertical lines interrupting the I_m trace result from ramp voltage commands to test current-voltage (*I-V*) functions. *C*, *H*, and *R* mark the ramp-command samples. (B) Current-voltage (I-V) plots illustrating "ohmic" resistance of glial membrane. Note right-shift of *I-V* plot during large increase of holding current, corresponding to strong negative shift of ΔV_0. Input resistances (determined from the slope of *I-V* plots): Control: 106 MΩ; Hypoxia: 116 MΩ (slightly later the input resistance decreased to 103 MΩ); Recovery: 114 MΩ. The patch pipette was filled with Cs-gluconate solution (for further technical details, see Czéh et al., 1992a). [From an experiment by G. Czéh; from Somjen et al. 1993, with permission.]

orous conditions of voltage clamp what has been observed earlier with conventional intracellular electrodes. During intense neuronal excitation, as well as under the extreme condition of spreading depression, glial cells depolarized with little or no change of membrane conductance. The most likely agent causing the depolarization is the rising $[K^+]_0$. Electrogenic transport of transmitter amino acids may also cause depolarization, but its influence should wane with rising $[K^+]_0$ (Barbour et al., 1988). The modest increase of membrane conductance may have been due to the action of transmitter agents spilling over from synaptic junctions and acting on glial receptors (MacVicar et al., 1989; Berger et al., 1992b; Clark and Mobbs, 1992; Jabs et al., 1993; Steinhäuser et al., 1993). An alternative explanation would be the opening of stretch gated channels (Kimelberg and Frangakis, 1986; Kimelberg and Kettenmann, 1990; Kimelberg et al., 1990a; Kempski et al., 1991; Puro, 1991; Bowman et al., 1992; Islas et al., 1993).

During spreading depression of Leão, glial cells undergo especially strong depolarization (Sugaya et al., 1964, 1975; Higashida et al., 1974). At the same time ΔV_0 and $\Delta[K^+]_0$ also assume unusually large magnitude. This has lead to speculation concerning a possibly leading role of glial cells in causing spreading depression. The absence of an early "active" membrane response by glia during spreading depression (Figure 20-8) weakens the suggestion of their primacy in generating this pathophysiological event (discussed in detail by Somjen et al., 1992; Herreras and Somjen, 1993).

NEWLY DISCOVERED CHANNELS, AND THEIR POSSIBLE ROLE IN SETTING GLIAL MEMBRANE POTENTIAL

The early literature has suggested two mechanisms by which glial cells could be depolarized during intense neuronal excitation: the direct effect of rising $[K^+]_0$, and electrogenic transport of neuroactive substances. Yet another potential depolarizing effect is cell swelling. At least in cultured astrocytes, a number of laboratories report depolarizing currents activated by hypotonic solutions (Kimelberg and Frangakis, 1986; Kimelberg and O'Connor, 1988; Kimelberg and Kettenmann, 1990; Kimelberg et al., 1990a; 1990b; Kempski et al., 1991; Puro, 1991; Bowman et al., 1992; Islas et al., 1993). There is evidence that glial cells do swell during neuronal excitation (Dietzel et al., 1989; Andrew and MacVicar, 1992; Osehobo et al., 1992). In these cases the swelling is assumed to be the result of the rising $[K^+]_0$ and the consequent uptake of ions and water (Ballanyi et al., 1987). The opening of swelling-activated channels could amplify the depolarization caused directly by the elevation of $[K^+]_0$.

Finally we come to the already mentioned abundance of ion channels and receptors that have recently been demonstrated. For the most part these channels were found in glial cells in culture, but some were demonstrated also in freshly isolated cells and also in tissue slices (MacVicar, 1984; Walz and MacVicar, 1988; Barres et al., 1990; Barres, 1991a; Berger et al., 1991, 1992a, 1992b; Marrero et al., 1991; Clark and Mobbs, 1992; Ransom and Sontheimer, 1992; Steinhäuser et al., 1992, 1993). Mudrick-Donnon et al. (1992) have found that the so-called stellate cells in the pars intermedia of the pituitary gland generate re-

sponses that, by all criteria, resemble $GABA_A$ and dopamine (D_2)-mediated synaptic potentials. Moreover, at least in some cells at certain stages of maturation, voltage steps also evoke "active" currents in whole cell configuration (Berger et al., 1991, 1992a; Steinhäuser et al., 1992, 1993; see Chapters 11 and 12, this volume). By and large, the older the animal from which the tissue slice is taken, and the more mature the cells in a culture, the more likely that the glial membrane responds to imposed voltage or current steps as if it was an "ohmic" resistor (Sontheimer et al., 1991; Steinhäuser et al., 1992, 1993), but the administration of transmitter agents opened ligand gated channels regardless of age (Barres, 1991a; Berger et al., 1992b; Clark and Mobbs, 1992).

CONCLUSIONS

We have found in the literature four distinct candidate mechanisms that could be responsible for the depolarization of glial cells during neuronal excitation:

1. The rise of $[K^+]_0$ in interstitial spaces during neuronal activity, which acts directly on the highly selective K^+ permeability of the glial membrane
2. The uptake of transmitter molecules by an electrogenic transport mechanism
3. The opening of stretch-activated channels during cell swelling
4. Ligand-gated channels

Although the four explanations are not mutually exclusive, it should be noted that rising $[K^+]_0$, while it is the very cause of the depolarization by the first mechanism, inhibits the second (electrogenic transport) (Barbour et al., 1988). Neither the first nor the second mechanism requires a change of membrane resistance, but the third and fourth ones do. Most writers, regardless of their adherence to one or other hypothesis, assume that the glial depolarization is subordinate to neuronal activity, and they do not assign a role in signalling or in information processing to glial cell depolarization.

The question remains, whether the inability to experimentally demonstrate the activation of ion channels in glial cells *in situ* refutes their operation in "real" brains, or whether this inability results from the inadequacy of experimental technique? Active currents have not been evoked either by depolarizing voltage or current steps. Only during intense neuronal seizure discharges and during spreading depression was there some increase of conductance of the glial membrane, but even that response remained feeble compared to that shown by neurons.

Two explanations have been offered to resolve the seemingly contradictory findings. It has been suggested that the conductance through the electrotonic coupling among glial cells shunts the membrane of individual glial cells. Changes of membrane conductance would therefore not be detected in recordings made from an individual cell, which is but a single element in an extensive randomly coupled net. Coupling among cells could indeed explain the apparently constant input resistance to voltage or current steps injected into a single cell. The same explanation is, however, not valid when the entire population of glial cells is depolarized during seizures and spreading depression. Both voltage- and ligand-gated channels of all members of the electrotonic net should be wide open under these conditions. The expected drop of resistance, spread over the surface of the entire quasi-syncytial assembly, should clearly be detectable by measurement in both current clamp and voltage clamp mode. Yet the input resistance of glial cells remains either stubbornly constant or changes only slightly in these extreme conditions.

Barres (1991b) offered another explanation for the apparently ohmic resistance, namely, that the complementary operation of inward and outward rectifying channels mutually cancel each other's effect. If this is the case, then perhaps the function of complementary inward and outward rectifying K^+-channels is to insure a high, selective, constant K^+-conductance at all membrane potential levels. Perhaps rectification is a fundamental property of specialized ion channels; perhaps it is not possible to build membrane channels that are selective for K^+, and yet conduct equally in both directions.

What then is the significance of the glial Na^+, Ca^{2+}, and Cl^- channels and of the receptors for a wide spectrum of neuroactive ligands? Speculation concerning the possible roles of these membrane constituents falls outside the scope of this chapter (see Chapter 12 for further discussion). Here we are limited to but one conclusion: the generation of glial electric signals is not among their functions.

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IV Receptors

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21 Gamma-aminobutyric acid and glutamate receptors

GABRIELA v. BLANKENFELD, KRISTIAN ENKVIST, AND HELMUT KETTENMANN

Glutamate and gamma-aminobutyric acid (GABA) are the most abundant transmitters in the central nervous system. They mediate excitation and inhibition, respectively. Released mainly at the presynaptic terminal, they can activate different types of receptors on the neuronal postsynaptic membrane. A number of recent studies, however, indicate that these specific receptors are also expressed by glial cells. In this chapter, the pharmacological and molecular properties, cellular distribution and speculations on the functional significance of glial GABA and glutamate receptors will be discussed. As will become apparent, this field of research is in a highly interesting phase: the molecular structure of the different $GABA_A$ and glutamate receptors composed of different subunits is now being elucidated. Molecular biologists have begun to learn that such receptor subunits are also present on glial cells. Thus, the picture of glial GABA and glutamate receptors is just emerging and is far from complete. Some basic facts, however, have been established: (1) glial cells can express GABA and glutamate receptors *in vitro* and *in situ* (2) there are similarities and differences between neuronal and glial receptors. These advances have not answered a key question: what is the role of glial GABA and glutamate receptors in the functioning of the brain? So far, only speculations can be offered.

METHODS USED TO STUDY GLIAL GABA AND GLUTAMATE RECEPTORS

This chapter covers several approaches to studying the properties of glial GABA and glutamate receptors. Binding studies with specific ligands indicate the presence of these receptors on glial cells. Antibodies directed against a specific domain of the receptor protein can serve to identify the individual subunits within the receptor complex in the plasma membrane and its distribution within the cell. *In situ* hybridization with specific primer sequences against mRNA coding for specific receptor subunits yields the expression pattern in different cell types and brain regions. The opening of ionotropic receptors can be directly approached with electrophysiological methods, such as the patch-clamp technique, which represents the most direct functional approach. Receptor-mediated increases in cytosolic ion concentrations or release from intracellular stores can also be monitored using optical indicators. Since activation of G protein-coupled receptors triggers a cascade of intracellular second-messenger systems, biochemical methods can also be used to study the pharmacological properties of the receptor. These studies involve investigating cell proliferation, release of substances, and activation of second-messenger pathways.

GABA RECEPTORS

Molecular Diversity

Most of the studies characterizing the biophysical and pharmacological properties of GABA and glutamate receptors in the central nervous system were performed on neurons. GABA can activate two main classes of receptors: the ionotropic $GABA_A$ and the metabotropic $GABA_B$ receptors. The $GABA_B$ receptor is coupled to a G protein. Activation of the receptor leads to an increase in K^+ conductance or a decrease in Ca^{2+} conductance of the membrane via second-messenger pathways. The molecular structure of the receptor protein is so far unknown, and there are only few reports on the expression of such receptors in glial cells: Binding of the specific $GABA_B$ receptor agonist baclofen was demonstrated in cultured astrocytes (Hösli and Hösli, 1990). The $GABA_A$ receptor contains an intrinsic ion channel mainly selective for Cl^-. The increased Cl^- conductance on receptor activation mediates inhibition in neurons. The $GABA_A$ receptor channel has a complex structure with different binding sites for barbiturates, benzodiazepines, and steroids. These substances can modulate the current flow through the receptor channel. Barbiturates and benzodiazepine agonists enhance, while inverse benzodiazepine agonists de-

crease GABA-induced responses (Sieghart, 1992). The modulation of the GABA-activated response by steroids can increase or decrease the response, depending on the type of steroid (Majewska, 1992). In analogy to the nicotinic acetylcholine receptor, the $GABA_A$ receptor is likely to be composed of five subunits. While a number of different subunits have been cloned, the composition of any type of native $GABA_A$ receptor is unknown. The α subunits (α_1 to α_6) most likely contain the benzodiazepine receptive site. In addition, a number of β (4), γ (3), and a δ subunit have been cloned (for review see Lüddens and Wisden, 1991; Sieghart, 1992).

Glial Responses to GABA Characterized in Cultured Cells

The first hints of the presence of $GABA_A$ receptors in glial cells came from studies of cultured glial cells. In these studies on primary cultures, the physiological results could be related to a certain cell type by the use of specific immunocytochemical markers (Gilbert et al., 1984; Kettenmann et al., 1984). Studies on cultured astrocytes from rat cortex described basic properties of astrocytic $GABA_A$ receptors, which revealed many similarities to neuronal $GABA_A$ receptors. The receptor contains an intrinsic Cl^- channel with similar (gross) single-channel properties as described for neuronal $GABA_A$ receptors (Bormann and Kettenmann, 1988) (see Figure 21-1B). Responses can be induced by the specific $GABA_A$ receptor agonist muscimol, and blocked by the Cl^- channel blocker picrotoxin and the antagonist bicuculline (Kettenmann and Schachner, 1985). The GABA-induced response can be enhanced by the barbiturate pentobarbital and by the benzodiazepine agonists diazepam or flunitrazepam. Steroid hormones can act as positive or negative modulators of the GABA-induced response (Chvatal and Kettenmann, 1991) (see Figure 21-1D). The dose-response curve revealed the presence of two allosteric GABA binding sites on the receptor complex (Bormann and Kettenmann, 1988) (see Figure 21-1C).

These studies in astrocytes, however, also demonstrated differences in comparison to neuronal $GABA_A$ receptors: the inverse benzodiazepine agonists such as the β-carboline DMCM enhanced the response in astrocytes in contrast to its inhibitory effect in neurons (Backus et al., 1988). The subunit composition of $GABA_A$ receptors in cultured astrocytes was analyzed in a study by Bovolin et al. (1992) with *in situ* hybridization techniques. The expression pattern of 14 subunits was studied in cultured granule cells as compared to cerebellar astrocytes. The total amount of mRNA was found to be two times higher in granule cells than in astrocytes. While mRNAs coding for the α_6 and the γ_2 subunit were not detected, mRNA for all other 12 subunits was found in the astrocytes. Prominent expression of the α_1, α_2, and γ_1 was found, the latter subunit could mediate the different DMCM sensitivity seen in patch-clamp experiments.

In cells of the oligodendrocyte lineage the inverse benzodiazepine agonist DMCM acted as in neurons, in contrast to the responses in astrocytes. However, the dose-response curve revealed no allosteric interaction between binding sites as described for astrocytic and neuronal $GABA_A$ receptors (Blankenfeld et al., 1991). These studies indicate that cultured glial cells express receptors with different pharmacological properties.

GABA Receptor Activation Depolarizes Glial Cells

In most adult neurons, the activation of $GABA_A$ receptors leads to a hyperpolarization of the membrane due to an influx of Cl^- into the cell. Some immature neurons, however, are depolarized by GABA, which is thought to act as an excitatory transmitter in early postnatal development (Cherubini et al., 1991). Independent of developmental stage or brain region, astrocytes and oligodendrocytes were always found to be depolarized by GABA. This is due to a different Cl^- distribution in neurons and glial cells. In glial cells the intracellular Cl^- concentration is increased far above the passive distribution, leading to an efflux of Cl^- as determined with ion-selective microelectrodes (Kettenmann et al., 1987; Hoppe and Kettenmann, 1989) (see Figure 21-1A). In oligodendrocytes, the Cl^- inward transport is primarily due to the activity of a bumetanide-sensitive $Na^+/K^+/Cl^-$ transport system. The mechanism of Cl^- transport across the astrocytic membrane additionally involves $Na^+/K^+/2Cl^-$ cotransport and Cl^-/HCO_3^- exchange (Kettenmann, 1990; Kimelberg, 1990). This difference in ion flux direction between adult neurons and glial cells could be of functional importance (as discussed below).

In glial precursor cells it was found that the GABA-induced depolarization activates voltage-gated Ca^{2+} channels (Kirchhoff and Kettenmann, 1992) (see Figure 21-2). The resulting increase of cytosolic Ca^{2+} could be a signal leading to physiological changes in glial precursor cells. In more mature oligodendrocytes, the depolarization is too small to activate the Ca^{2+} channels (see Figure 21-2).

New Insights from Studies on Glial Cells In Situ

The cell culture studies described above demonstrated that glial cells have the capacity to express

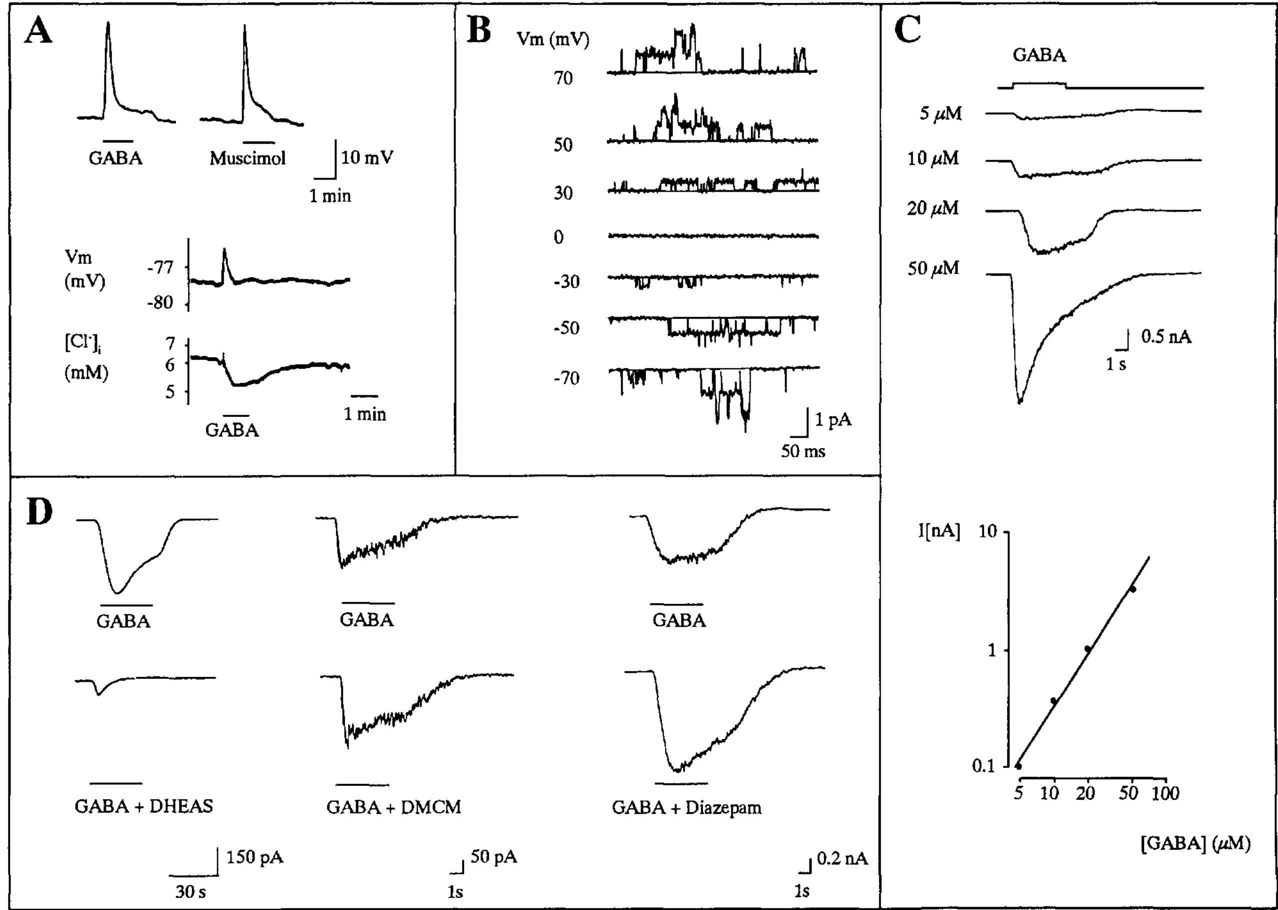

FIG. 21-1. Properties of $GABA_A$ receptors in cultured astrocytes. **(A)** Effect of 1 mM GABA and muscimol on the membrane potential of a cultured cortical astrocyte, as recorded with intracellular microelectrodes. Muscimol induced a similar depolarization as GABA, indicating the activation of $GABA_A$ receptors [*upper traces*: from Kettenmann and Schachner (1987), with permission.] Using an ion-selective recording technique, the intracellular Cl^- concentration was determined while simultaneously monitoring the membrane potential, in a cultured oligodendrocyte from the spinal cord [*lower trace*: from Hoppe et al. (1989), with permission.] The traces demonstrate that the depolarization is caused by Cl^- efflux. **(B)** Single-channel currents activated by GABA were recorded with the patch-clamp technique in the outside-out configuration from a cultured astrocyte at different membrane potentials. With symmetrical Cl^- and uneven Na^+ K^+ distribution, single-channel currents reversed polarity at about 0 mV, the Cl^- equilibrium potential. The single-channel conductance calculated from the resulting voltage-curve was 29 pS. [From Bormann and Kettenmann (1988), with permission.] **(C)** Whole-cell current recordings from a cultured astrocyte in response to increasing concentrations of GABA (*upper trace*). In the graph below, inward currents are plotted on a double-logarithmic scale versus the GABA concentration. The slope of the dose-response relationship of the GABA-induced currents is about 1.6, indicating allosteric interactions between two ligand binding sites. [From Bormann and Kettenmann (1988), with permission.] **(D)** Modulation of $GABA_A$ receptor channels in cultured astrocytes by agonists to the steroid or benzodiazepine binding site. The steroid DHEAS decreased the GABA-activated currents. [From Chvatal et al. (1991), with permission.] DMCM, and diazepam, an inverse and full agonist, respectively, both increased the GABA-induced current response. [From Bormann and Kettenmann (1988), with permission.]

GABA and glutamate receptors. No information was available about the distribution of GABA receptors in glial cells in respect to brain area and cellular microenvironment. This question can now be addressed owing to recent advances in physiological and molecular biological techniques. With the application of the patch-clamp method to thin-slices, glial cells can be studied in an intact environment. In the following preparations, glial GABA receptors were investigated with this technique: in astrocytes of the retina (Malchow et al., 1989; Clark and Mobbs, 1992) and hippocampus (Steinhäuser et al., 1994), in glial precursor cells and oligodendroctyes of the corpus callosum (Berger et al., 1992), and in Bergmann glial cells of the cerebellum (Müller et al., 1993a). Antibodies specific for subtypes of $GABA_A$ receptor subunits gave some information about the subunit composition of glial GABA receptors. In particular, studies on two types of glial cells have increased our knowledge about the properties of

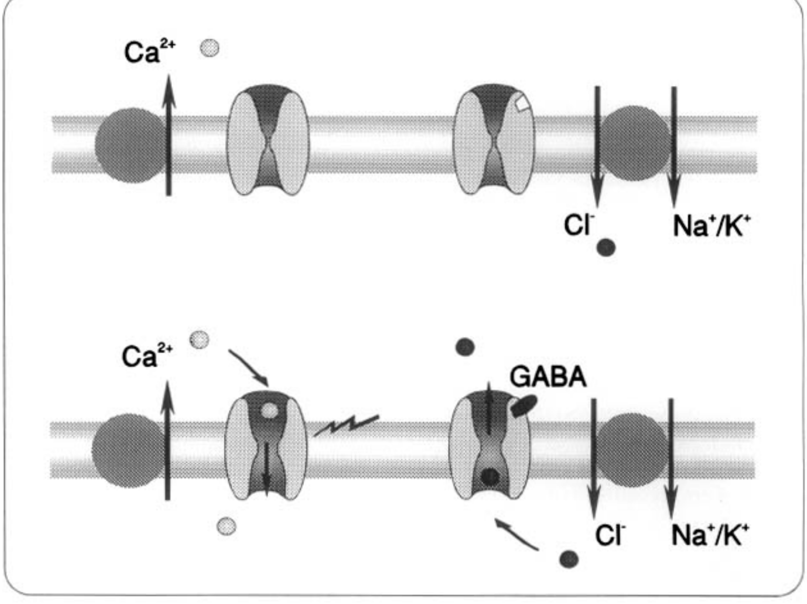

FIG. 21-2. Developmental changes in GABA-induced Ca^{2+} movements. The development of oligodendrocytes from their precursor cells is accompanied by morphological changes and a change in the antigenic phenotype (*upper diagram*). A2B5-positive, immature precursor cells express $GABA_A$ receptors and two types of voltage-activated Ca^{2+} channels. The activation of the GABA receptor leads to an influx of Ca^{2+} through these channels, as shown in the *lower trace* [from Kirchhoff and Kettenmann (1992), with permission.] Whereas O4-positive precursor cells and O1-positive oligodendrocytes have no voltage-activated Ca^{2+} channels, the O10 positive oligodendrocyte express Ca^{2+} channels, but only a few $GABA_A$ receptors. The activation of these $GABA_A$ receptors is not sufficient to activate the Ca^{2+} channels and trigger a Ca^{2+} flux. The following mechanisms (most likely) link $GABA_A$ receptor activation and activation of voltage-gated Ca^{2+} channels: the internal Cl^- in oligodendrocytes is elevated above the passive distribution, by the action of a bumetanide-sensitive $Cl^-/Na^+/K^+$ cotransporter. $GABA_A$ receptor activation opens the Cl^- channel, leading to an efflux of Cl^- and a resulting depolarization of the membrane. The depolarization activates voltage-gated Ca^{2+} channels, and triggers the flux of Ca^{2+} into the cell.

glial GABA receptors *in situ*; Bergmann glial cells, and oligodendrocytes.

In an *in situ* hybridization study on Bergmann glial cells, mRNA coding for the α_2 and the γ_1 subunit was found (Wisden et al., 1989). Studies with monoclonal antibodies against the different receptor subunits showed the α_2 and the δ subunit to be part of the receptor complex (Müller et al., 1994). The widespread γ_2 subunit, which confers benzodiazepine sensitivity to a $GABA_A$ receptor complex, could not be demonstrated using *in situ* hybridization (Wisden et al., 1989) or immunohistochemistry (Müller et al., 1994). Indeed, electrophysiological studies verified that Bergmann glial cells were unresponsive to benzodiazepines. Moreover, activation of $GABA_A$ receptors not only opens Cl^- channels, but also transiently induces a blocking of K^+ channels. The molecular mechanisms leading to the reduction of the K^+ conductance is not understood (Müller et al., 1994).

Glial cell processes in white matter tracts of adult cats could be stained with polyclonal antibodies

against the β_1 subunit. No immunoreactivity was found on microglial or oligodendroglial cell bodies (Gu et al., 1992). In thin slices of the corpus callosum the expression of $GABA_A$ receptors on glioblasts and oligodendrocytes could be verified with electrophysiological methods (Berger et al., 1992).

Developmental Regulation

Tissue culture studies have indicated a developmental regulation of $GABA_A$ receptor expression in the oligodendrocyte lineage. The density of $GABA_A$ receptors on glial precursor cells was about 100 times higher, as compared to oligdendrocytes (Blankenfeld et al., 1991). A similar tendency was also observed for glioblasts and oligodendrocyte in the corpus callosum (Berger et al., 1992). While the receptor expression in cells of the oligodendrocyte lineage decreased continuously during development, $GABA_A$ receptor expression was transient in Bergmann glial cells: it was maximal during the time period of granule cell migration along the Bergmann glial processes to the inner granule cell layer. At that developmental period, the Purkinje cells also form contacts with the parallel fibers. In the adult animal, Bergmann glial cells ensheath the dendrites of the Purkinje cells (Müller et al., 1994). This precise developmental timing of the expression of the receptor leads to the speculation that glial $GABA_A$ receptors have a synchronizing function during these developmental events.

GLUTAMATE RECEPTORS

The glutamate receptors form a family of receptors with different properties. Ionotropic NMDA and AMPA-kainate receptors, and the metabotropic glutamate receptors are composed of a number of subunits that introduce different properties to the receptor complex.

Activation of the ionotropic AMPA-kainate receptor leads to the opening of a cation channel which depolarizes the membrane. In neurons this event mediates excitation. Among the ionotropic receptor class, a number of subunits that make up the functional receptor, either as a homomer or heteromer, have been cloned (for review see Nakanishi, 1992; Sommer and Seeburg, 1992). The GluR2 subunit appears to be particularly important for the properties of the receptor channel. If this subunit is present, the receptors have a near-linear current-voltage relationship, and low Ca^{2+} permeability. In the absence of the GluR2 subunit the channels display rectification and are significantly permeable to Ca^{2+} in addition to the Na^+/K^+ permeability.

Metabotropic glutamate receptors resemble other G protein-coupled receptors in the plasma membrane, with an extracellular NH_3 terminal and seven transmembrane segments. Like the ionotropic receptors, they can be divided into subgroups (for review see Nakanishi, 1992). Functionally, metabotropic glutamate receptors are coupled to breakdown of inositol phospholipids, leading to activation of PKC by diacylglycerol, and mobilization of Ca^{2+} from intracellular stores by IP_3. Subtypes coupled to a decrease in cAMP and cGMP levels have also been reported.

Ionotropic Glutamate Receptors in Cultured Glial Cells

As with the $GABA_A$ receptor, our first knowledge of the glial glutamate receptor came from studies of cultured glial cells (see Figure 21-3). Using electrophysiological techniques Bowman and Kimelberg (1984) and Kettenmann et al. (1984) demonstrated depolarizations induced by glutamate in cultured astroglia. The agonists kainate and quisqualate, but not NMDA, mimicked the glutamate response hinting to the presence of AMPA-kainate receptors, as demonstrated by electrophysiological methods (Bowman and Kimelberg, 1984; Kettenmann et al., 1984; Kettenmann and Schachner, 1985) or by monitoring [^{3}H]GABA release (Gallo et al., 1989). Receptor activation leads to an opening of a cationic pore like in neurons (Sontheimer et al., 1988), with concomitant influx of Ca^{2+} from the extracellular space (Enkvist et al., 1989). Glutamate receptors have been demonstrated in a number of different cell culture systems, including type I and II astrocytes and oligodendrocytes (for review see Blankenfeld and Kettenmann, 1992).

The glutamate receptor in cerebellar process-bearing astroglia has been characterized at the single-channel level and possesses at least five subconductance levels, ranging from 6 to 47 pS, unlike kainate receptors in neurons (Usowicz et al., 1989). In some culture systems, glutamate receptor currents have been recorded only after at least 1 week in culture, indicating that environmental factors can control their expression (Wyllie et al., 1991). The presence of a kainate binding protein, presumed to be part to a kainate receptor, was first demonstrated by Gregor et al. (1989) in cultured chick Bergmann glia.

Metabotropic Glutamate Receptors in Cultured Glial Cells

Evidence for the expression of metabotropic glutamate receptors in cortical astroglial cells was first presented by Pearce et al. (1986). They demon-

strated increases in both the breakdown of inositol phospholipids and $^{45}Ca^{2+}$ efflux upon stimulation with glutamate receptor agonists. The efficacy was in the order quisqualate ≥ glutamate > kainate >> NMDA, which is the expected pharmacology of the metabotropic quisqualate receptor subtypes mGluR1 and R5. This finding was substantiated by Enkvist et al. (1989) with the use of the optical indicator fura-2, demonstrating both Ca^{2+} influx and Ca^{2+} release from intracellular stores in cultured cortical astroglia in response to glutamate and quisqualate (see Figure 21-3). Glutamate-induced oscillations in cytosolic Ca^{2+} in single-cultured astroglia were described by Glaum et al. (1990) and Jensen and Chiu (1990) using optical indicators. The increases in cytosolic Ca^{2+} triggered by activation of the metabotropic receptor are thought to be involved in generating Ca^{2+} waves spreading through an astrocytic syncytium (Cornell-Bell et al., 1990a; see Chapter 10, this volume).

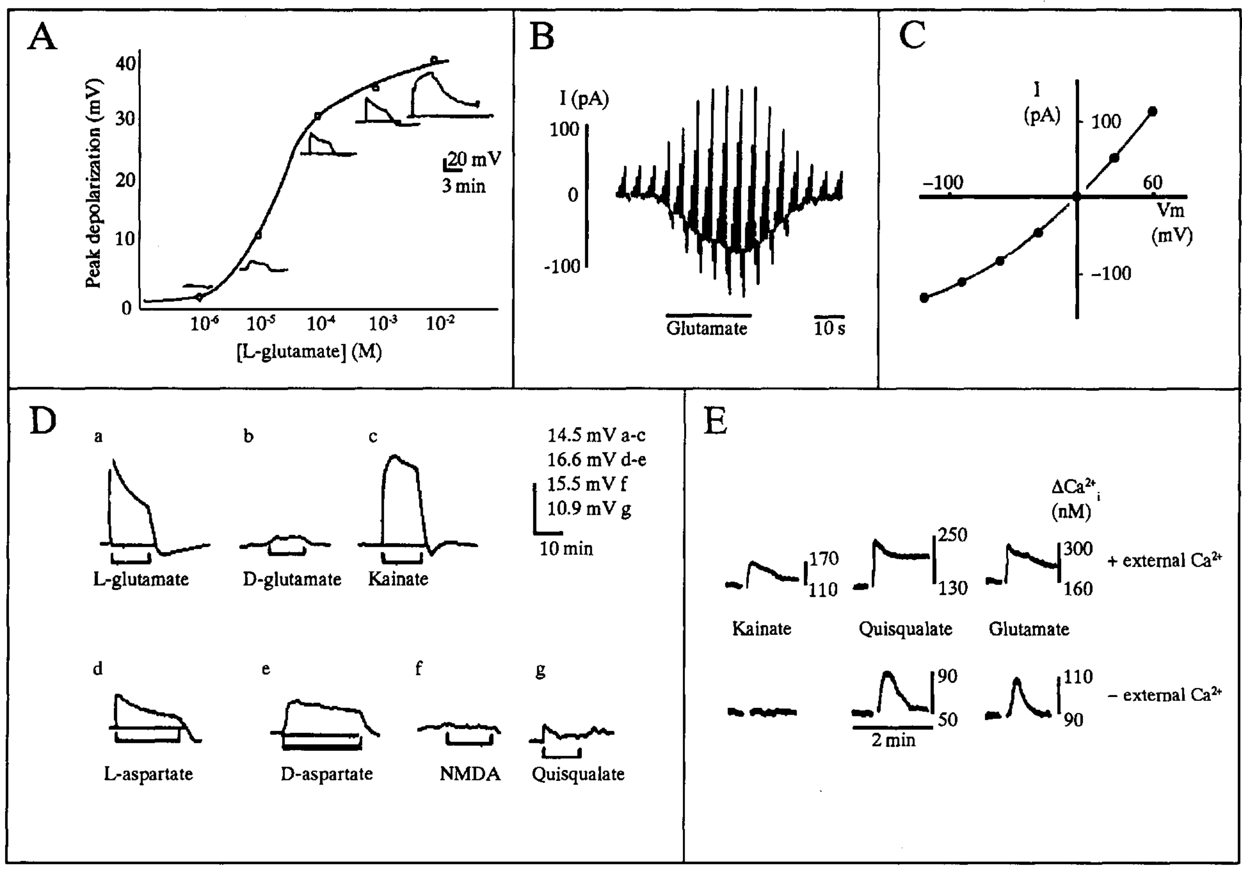

FIG. 21-3. Properties of the glutamate receptor in cultured astrocytes. **(A)** Depolarizing responses to increasing concentrations of L-glutamate. Initial peak depolarizations are plotted versus agonist concentration. The dose-response curve was obtained from a single-cultured astroglial cell, using an intracellular microelectrode measuring the membrane potential, and applying increasing concentrations of agonist. The *insets* show responses to the indicated concentrations. [From Bowman and Kimelberg (1984), with permission.] **(B)** Patch-clamp recordings from a single astroglial cell. The membrane current was measured while briefly clamping the cell's membrane potential to different values, from a resting potential of −60 mV to −120, −90, −30, 0, 30, and 60 mV. The duration of the voltage pulse was 200 ms, with a 1-second interval between pulses. The application of glutamate in the bathing medium significantly increased membrane currents, indicating the opening of an ion channel. [From Sontheimer et al. (1988), with permission.] **(C)** Current voltage curve obtained from the measurements in Figure B. The measured current at each of the voltage steps under control conditions is subtracted from that measured after the application of glutamate. The current voltage curve reverses at a membrane potential of 0 mV as expected for a cationic conductance and is similar to neuronal AMPA-kainate receptors. [From Sontheimer et al. (1988), with permission.] (D) Pharmacology of astroglial responses to excitatory amino acids. The membrane potential of cultured astroglial cells was measured with an intracellular microelectrode. All applications are 10^{-4} M. This pharmacological profile identifies the receptor as an AMPA-kainate type glutamate receptor. [From Bowman and Kimelberg (1984), with permission.] (E) Increases in intracellular Ca^{2+} measured with the optical indicator fura-2. All applications are 5×10^{-4} M. In the absence of extracellular Ca^{2+}, quisqualate and glutamate induce a transient increase in intracellular Ca^{2+}, suggesting release from intracellular stores. Kainate only causes a Ca^{2+} increase in Ca^{2+}-containing extracellular buffer, indicating influx of this ion. This pattern of Ca^{2+} responses is expected in cells expressing both metabotropic and ionotropic receptors. [From Enkvist et al. (1989), with permission.]

Glutamate Receptors in Intact Tissue

The presence of glutamate receptors was recently demonstrated in acutely isolated slices, using both electrophysiology and *in situ* hybridization. With that approach the presence of glutamate receptors was verified in cells of the oligodendrocyte lineage in the corpus callosum (Berger et al., 1992), Bergmann glial cells from the cerebellum (Müller et al., 1992), glial cells from the hippocampus (Steinhäuser et al., 1994) and astrocytes from the rabbit retina (Clark and Mobbs, 1992). Patch-clamp studies in Bergmann glial cells revealed that this ionotropic glutamate receptor channel is Ca^{2+}-permeable and has a nonlinear current voltage relationship (see Figure 21-4). *In situ* hybridization showed the expression of the GluR1 and GluR4 subunits, but not of the GluR2 in these cells (Monyer et al., 1991). Recombinant receptors lacking the GluR2 subunit displayed the same properties as those in the Bergmann glial cells in thin slices and in cultured fusiform glial cells from the cerebellum (Burnashev et al., 1992). The study on Bergmann glia in intact slices also revealed that glutamate receptor activation is accompanied by a transient reduction of the resting K^+ conductance, most likely mediated by the influx of Ca^{2+} (Müller et al., 1992). A similar effect on the K^+ conductance was also observed in glial cells from the hippocampus (Steinhäuser et al., 1994). The physiological implications of this link

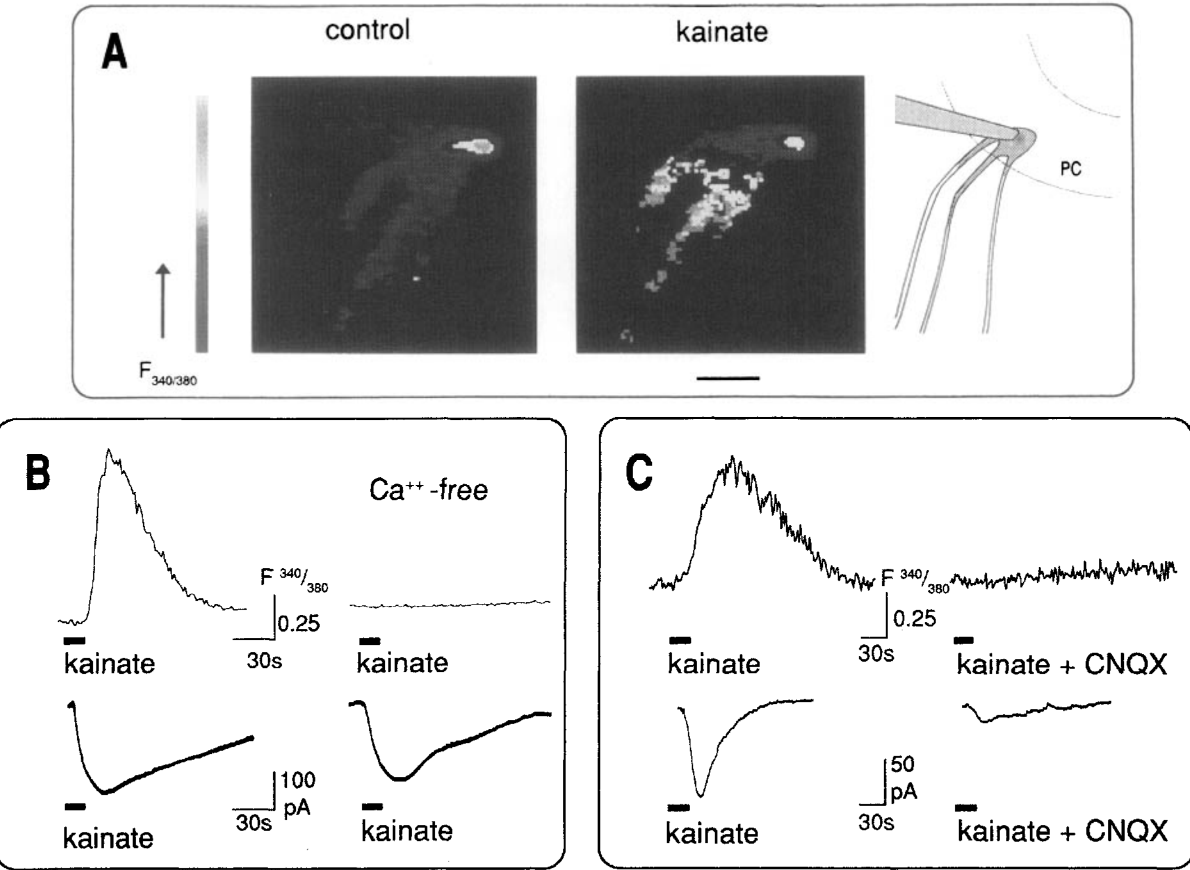

FIG. 21-4. Responses to kainate in Bergmann glial cells in an acutely isolated cerebellar slice. (A) A cell was filled with the calcium indicator dye fura-2 by loading from the patch-clamp pipette, and intracellular Ca^{2+} concentration was monitored on a video-based imaging system. The gray scale to the *left* of the pictures represents intracellular Ca^{2+} levels. The diagram on the *right* shows the cell with the pipette still attached to the soma. Three processes can be distinguished. The bar denotes 20 μm. The images illustrate the increase in intracellular calcium seen during the application of 1 mM kainate. (B) Effects of low extracellular calcium concentration. The *upper traces* show the fluorescence ratio at 340/380 nm excitation of fura-2. An upward deflection of the curve denotes an increase in intracellular calcium. The *lower traces* show simultaneous recording of membrane currents using whole cell voltage clamp. A downward deflection of the curve indicates an inward current. In the calcium-free solution no increase in intracellular calcium is seen, while the kainate-induced currents remain unaffected. This suggests that the calcium response is due to the influx of this ion from the extracellular space. (C) Responses to kainate, both intracellular calcium and membrane currents, are blocked by the ionotropic glutamate receptor antagonist CNQX. [From Müller et al. (1992), with permission.]

between glutamate receptor activation and K^+ channel activity are not understood, but it will clearly influence the K^+ buffering properties of the glial cell (see Chapter 10, this volume).

Responses to NMDA

Cultured glial cells have not been reported to respond to NMDA. However, responses to NMDA have been recorded in Bergmann glial cells in cerebellar slices and glial cells of the hippocampal slice (Steinhäuser et al., 1994; Müller et al., 1993). The current response is linked to an increase in membrane conductance associated with an increase in current noise (Müller et al., 1993). Surprisingly, these responses did not lead to a change in cytosolic Ca^{2+} and were not affected by the presence of Mg^{2+} or glycine as expected from the behavior of the neuronal NMDA receptors. So far, the molecular properties of glial NMDA receptors have not been described.

FUNCTIONAL CONSEQUENCES OF GABA AND GLUTAMATE RECEPTOR ACTIVATION

Recently, we have learned that glial cells have the capability to express distinct receptors for GABA and glutamate, and we have described their basic biophysical and pharmacological properties. However, the functional importance of these receptors on glia remains unknown. In the following sections, some data on physiological responses in glial cells to GABA and glutamate agonists are presented together with some speculations about possible functions.

GABA Receptors May Be Involved in Cl^- Homeostasis

In neurons, activation of $GABA_A$ receptors commonly leads to an influx of Cl^- into the cell, resulting in hyperpolarizing inhibitory postsynaptic potentials (ipsp), in contrast to glial cells where the membrane is depolarized. Since astrocytic processes are found in close vicinity to synapses, the efflux of Cl^- from adjacent astrocytic processes through GABA-activated Cl^- channels could serve to maintain a constant extracellular Cl^- at the synaptic cleft (Bormann and Kettenmann, 1988; MacVicar et al., 1989). The astrocytic $GABA_A$ receptor may thus serve to buffer extracellular Cl^- levels at synaptic regions. This would comprise an additional function of astrocytes, that of providing Cl^- homeostasis, besides their presumed involvement in the regulation of extracellular K^+.

Astrocytic Swelling by Glutamate Receptor Activation

Glutamate application causes astrocytes to swell. Since astrocytic swelling is an important event after head trauma leading to neurological impairment (see Chapter 10, this volume), the control of astrocytic volume is of high clinical importance. Smaller glial volume changes could also influence the volume of the extracellular space around neurons and thus modulate neuronal activity. Increases in astrocytic cell volume in response to glutamate was reported by several investigators (for review see Kimelberg, 1992). The effect of kainate on astrocytic swelling makes it likely that AMPA-kainate receptors are involved since kainate is not used as substrate for the glutamate uptake carrier (Kimelberg et al., 1989). Chan et al. (1990) observed that glutamate, aspartate, and quisqualate, but not kainate application resulted in the swelling of cortical astroglia. Interestingly, the swelling was antagonized by the NMDA receptor antagonists ketamine and MK-801 (Chan and Chu, 1989; Chan et al. 1990), which clearly demonstrates that the effect of glutamate on glial cells is more complex than expected from the pharmacology of the electrical and Ca^{2+} responses.

Control of Proliferation by Glutamate

Glutamate has also been shown to affect the proliferation of cortical astroglia as determined by [^{3}H]thymidine incorporation (Condorelli et al., 1989; Nicoletti et al., 1990), without affecting the overall viability of the cells. The pharmacology suggested the involvement of a metabotropic glutamate receptor. This was further supported by the finding that excitatory amino acids reduced the thymidine incorporation with a similar pharmacological profile as their effect on phosphoinositide lipid hydrolysis (Nicoletti et al., 1990).

Synaptic Activity Could Influence Glial Cells in the Vicinity via Glial Receptor Activation

In cultured hippocampal astroglia, Cornell-Bell et al. (1990b) demonstrated that application of glutamate induced a rapid formation of filopodia. The pharmacology of this response was compatible with the activation of non-NMDA receptors, since it could be mimicked by both quisqualate and kainate, but not

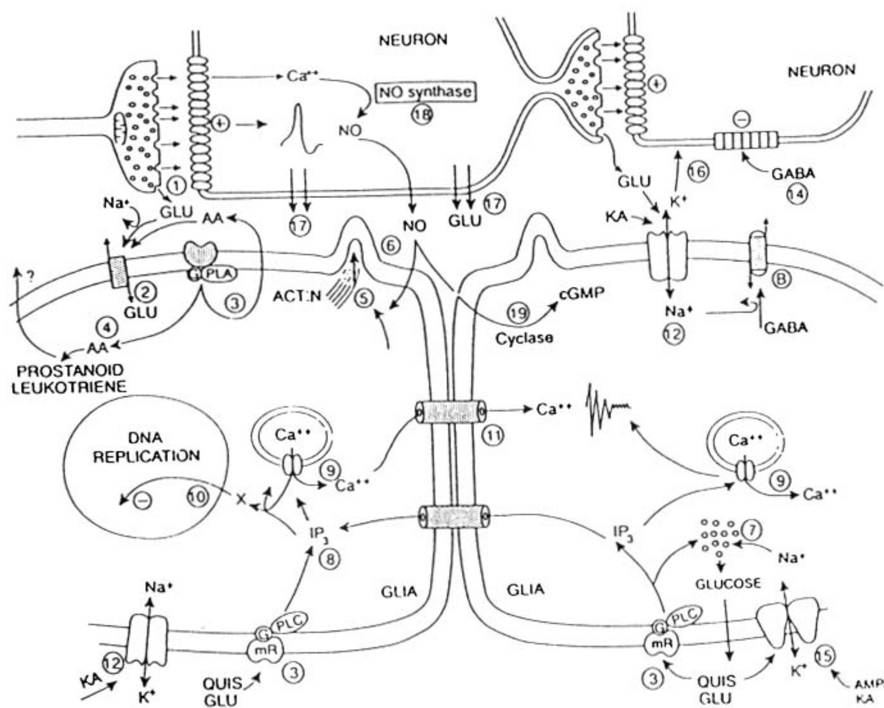

FIG. 21-5. Scheme of glutamatergic signaling pathways in neurons and glia. (*1*) Glutamate (GLU) release and postsynaptic excitation. (*2*) Na^+ dependent GLU uptake and its inhibition by arachidonic acid (AA). (*3*) Activation of the GLU metabotropic receptor to reduce (*4*) AA, (*5*) actin reorganization, (*6*) filopodia formation, (*7*) glycogenolysis, (*8*) IP_3 formation, (*9*) calcium release, and (*10*) inhibition of replication. (*11*) Permeation through gap junctions and calcium waves. (*12*) Activation of ionotropic receptors by GLU, influx of Na^+ and efflux of K^+. (*14*) Extrasynaptic inhibition by GABA. (*15*) Activation of AMPA receptors by GLU. (*16*) K^+ induced depolarization. (*17*) Axonal release of GLU. (*18*) NMDA receptor activation, calcium entry and stimulation of NO synthase. (*19*) Diffusion of NO from the neuronal compartment to the glial compartment and stimulation of guanylate cyclase and cGMP formation. [From Teichberg (1991), with permission.]

by NMDA. The authors concluded that the filopodia formation is mediated by inositol phospholipid hydrolysis, resulting in a reorganization of the cytoskeleton.

It can also be speculated that *in vivo* synaptic release of these neurotransmitters could activate transmitter receptors on glial cells and trigger extension of glial processes. The presence of glial processes around a synapse could be important for synaptic stability. Survival or annihilation could be mediated by glial activity triggered by the synapse itself. The concept for such a neuron-glia interaction is strengthened by the observation that the transplantation of astrocytes into the adult cat visual cortex reestablished ocular dominance plasticity (Müller and Best, 1989). Neuron-glia interactions at synaptic regions could thus be important for long-term modification of signal transduction, resulting in complex functions such as "memory." The involvement of glial transmitter receptors in such a neuron-glia interaction could even involve complex, interactive processes. For instance, nitric oxide released from neurons upon NMDA receptor stimulation could activate glial guanylate cyclase (Garthwaite, 1991). GABA is released from glial cells on glutamate receptor stimulation (Gallo et al., 1989) and could inhibit neuronal signaling. These possible, complex interactions are summarized in the Figure 21-5 diagram by Teichberg et al. (1991).

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22 Acetylcholine and serotonin receptor activation

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Progress in studying the roles of acetylcholine and serotonin in neuronal-glial interactions has not been as dramatic as in the cases of glutamate and GABA (see Chapter 21, this volume). Indeed, the best evidence for such interactions comes from invertebrate nervous systems rather than mammalian preparations. The reason may be that both acetylcholine and serotonin are less widely used in synaptic transmission than glutamate and GABA. In mammals, cholinergic neurons are localized in discrete nuclei in lower regions of the brain, sending their axons throughout the neocortex. Most serotonergic neurons are located in the raphe nuclei, but some other areas contain them. However, there is an extensive, patternless innervation of the cerebral cortex. Interestingly, most of the serotonergic varicosities are characterized by nonsynaptic contacts (Ridet et al., 1993). The proportion of astrocytes around these varicosities is quite high. The distance between these varicosities and astrocytes is about twice as large as that between synaptic vesicles in serotonergic synapes and astrocytes. The possibility that serotonin may not only be released by synapses but also by varicosities opens interesting possibilities of interactions between these serotonergic pathways and neighboring astrocytes.

ACETYLCHOLINE

Effects of Acetylcholine on Invertebrate Glial Cells

Neuropile Glial Cells of the Leech. Ballanyi and Schlue (1989) investigated the effect of acetylcholine receptor activation on the physiological properties of neuropile glial cells in the leech central nervous system. Application of acetylcholine elicits a small depolarization. The amplitude can be increased severalfold after exposure to acetylcholine esterase blockers. In the presence of these blockers the concentration threshold for acetylcholine was below 5 μM. Carbachol is less potent than acetylcholine (in the presence of these inhibitors), whereas nicotine is more potent than acetylcholine. The peak depolarizations reached 40 mV. A pharmacological profile of the response revealed a nicotinic receptor subtype (Ballanyi and Schlue, 1989). In such a nicotinic receptor, the ion channel is part of the receptor. Ballanyi and Schlue (1989) analyzed the ion movements underlying receptor activation with ion-sensitive microelectrodes. They found intracellularly a decrease in K^+ activity and an increase in Na^+ and Cl^- activity, as well as a transient acidification. These observations are compatible with a cation channel opening by receptor activation. Cl^- moves in passively and the acidification is probably due to Na^+/H^+ carrier inhibition, because of the reduced Na^+ driving force. Another interesting feature discovered by Ballanyi and Schlue (1989) is the permeability of the acetylcholine-gated channel for cholinergic agents such as choline, tetramethylammonium, and decamethonium. Since acetylcholine is a transmitter in the central nervous system of the leech, the authors suggest that receptor activation leads to K^+ uptake, and therefore contributes to extracellular K^+ buffering. It also can lead to choline uptake into glial cells by movement through the channel, once acetylcholine is broken down by acetylcholine esterase.

Schwann Cells of the Squid and Crayfish. In contrast to other vertebrate and invertebrate glia, the Schwann cells of these species (see Chapter 6, this volume) have low membrane potentials (−40 mV) and respond to axonal stimulation with hyperpolarizations (Villegas, 1984). This Schwann cell hyperpolarization is nicotinic receptor-mediated and K^+ alone is not a sufficient signal to induce the hyperpolarization. The underlying events are a complex mechanism involving two transmitter substances, one of which, acetylcholine, is released by the Schwann cells. Glutamate is stored in a cytoplasmic concentration of about 25 mM in the squid axons and released into the periaxonal space during activity (Lieberman, 1991). Glutamate interacts with several receptor subtypes of the Schwann cell membrane, but only the kainate/quisqualate subreceptor is of interest for this interaction (Evans et al., 1991). Lieberman and

Sanzenbacher (1992) suggested that the receptor activation results in an IP_3-mediated release of Ca^{2+} from intracellular stores into the cytoplasm of the Schwann cell. In these Schwann cells, acetylcholine is stored at a concentration 40 times higher than in the axoplasm (Villegas and Jenden, 1979). Heumann et al. (1981) showed that the Schwann cells are capable of synthesizing acetylcholine. The glutamate-mediated increase in intracellular Ca^{2+} leads to release of acetylcholine from the Schwann cells (Lieberman et al., 1989). This released acetylcholine acts in an autocrine fashion on the Schwann cells themselves via nicotinic receptors. Evans et al. (1985) showed that this receptor activation leads to an increase in cAMP levels via stimulation of adenylyl cyclase. Lieberman (1991) found that cAMP decreases a Cl^- conductance in the Schwann cell membrane, which leads to a long-lasting hyperpolarization. It is assumed that this hyperpolarization assists the Schwann cells in K^+ clearance of the periaxonal space, since the whole cascade is triggered by axonal activity. An obvious effect would be that the reduction in Cl^- conductance increases the relative K^+ permeability, so that the spatial buffer mechanism can work more effectively (see Chapter 47, this volume).

Effects of Acetylcholine on Vertebrate Astrocytes

Receptor Types and Distribution. *In vivo,* about 20% of the astrocytes in the rat brain label for muscarinic receptors. These cells are mainly found in the more superficial strata of the cortex and in the corpus callosum (Van der Zee et al., 1993). There was a correlation between GFAP (glial fibrillary acidic protein) expression and muscarinic receptor occurrence, suggesting that GFAP and muscarinic receptor expression are functionally linked to each other. Astrocytes in gray and white matter showed no differences in this respect. The study also found an increase in muscarinic receptor expression in astrocytes in aged animals.

It was found some time ago that virtually all cultured astrocytes and glioma cells possess acetylcholine receptors (Hamprecht et al., 1976; Repke and Maderspach, 1982). Repke and Maderspach (1982) found muscarinic receptors on astrocytes from chicken cerebral cortex. Murphy et al. (1986) found two muscarinic subtype receptors in cultured astrocytes: M1 and M2. The presence of an M1 subreceptor was inferred from a pirenzepine-inhibited increased turnover of membrane inositol phospholipids. The M2 receptor involvement was suggested by an inhibitory effect on adenylyl cyclase that was not inhibited by pirenzepine.

There is some evidence for nicotinic receptors. Hösli and Hösli (1988) found both nicotinic and muscarinic receptors on astrocytes in organotypic cultures of rat spinal cord and brainstem. The authors used autoradiographic studies that were subsequently confirmed by electrophysiological recordings (see below). They found that many of the astrocytes had muscarinic receptors. A smaller population of the astrocytes had nicotinic receptors. The nicotinic labeling was much weaker than the muscarinic one. Electrophysiological experiments (Hösli et al., 1988) revealed that a single astrocyte could possess both muscarinic and nicotinic receptors.

Activation of Signal Transduction Systems. Murphy et al. (1986) found evidence that M2 subreceptors inhibit the isoproterenol-induced stimulation of adenylyl cyclase. Enkqvist et al. (1989) measured a stimulation of transmembrane Ca^{2+} influx if the astrocytes were exposed to carbachol. In addition, activation of M1 subtype receptors on astrocytes stimulates phospholipase C (Murphy et al., 1986). This will create IP_3 and DG (diacylglycerol). An IP_3 increase will lead to Ca^{2+} mobilization. Such a Ca^{2+} mobilization, in addition to a transmembrane Ca^{2+} influx, was measured by Enkvqist et al. (1989) with the fura-2 method in response to carbachol exposure. An increase in the DG concentration would lead to protein kinase C activation. Furthermore, DeGeorge et al. (1986) reported arachidonic acid production, together with an increase in phosphoinositol metabolism after exposure to acetylcholine. This reflects the activation of the phospholipase C. Gustavsson et al. (1993) found that muscarinic receptor activation leads to phospholipase D activation. Phospholipase D is thought to be more involved in sustained responses of cells through the activation of protein kinase C, whereas Ca^{2+} mobilization is usually transient (Asaoka et al., 1992).

Electrophysiological Effects. Hamprecht et al. (1976) measured the membrane potential in the rat glioma cell line C6. The average resting potential was −28 mV, which is due to a high relative Cl^- conductance (Picker et al., 1981). The application of acetylcholine caused a hyperpolarization in all cells tested. This hyperpolarization was inhibited by atropine but not by D-tubocurarin, suggesting a muscarinic mechanism. However, α-bungaratoxin inhibited the response as well, implicating a nicotinic receptor and contradicting the finding of an underlying muscarinic receptor activation.

Hösli et al. (1988) used organotypic cultures from the spinal cord and brainstem to record membrane potential responses from astrocytes. If acetylcholine was applied, 86 cells hyperpolarized, 22 depolarized, and 27 showed no potential change. The amplitudes

of the responses were between 2 and 8 mV. The authors could not find any dose-response relationship between acetylcholine concentration and amplitude of the response. If muscarine or nicotine was used instead of acetylcholine, there was a similar pattern of hyperpolarization or depolarization and no clear dose-response relationship. When tested on the same astrocyte, acetylcholine, muscarine and nicotine always produced the same effect (either a hyperpolarization or a depolarization). Atropine blocked the muscarinic hyperpolarization, but not the nicotinic. Mecamylamine (a nicotinic antagonist) blocked the hyperpolarization caused by nicotine application. These findings seem to support an earlier autoradiographic demonstration of both nicotinic and muscarinic receptors on these cells (see above). However, the lack of a dose-response relationship and the small amplitudes involved throw the pharmacological validity of the responses into question. No other reports on cholinergic agonist-evoked potential changes or currents in cultured astrocytes are available.

Effect on Cell Functions. From their experiments with invertebrate glial cells, Ballanyi and Schlue (1989) suggest that a nicotinic receptor channel is permeable to choline and that such a channel assists in choline removal (and release) from glial cells. Since there are a few reports of nicotinic receptors in vertebrate astrocytes (see above), such a possibility is worth considering. There is no doubt that astrocytes are capable of choline uptake (see Massarelli et al., 1986). However, a kinetic evaluation of the uptake is compatible with high- and low-affinity carrier systems that appear to be Na^+-dependent (Wong et al., 1983). In general the uptake of choline into glial cells shows characteristics similar to those observed in neurons (for review see Massarelli et al., 1986). There is, however, one report on choline release from glial cultures upon stimulation by *muscarinic* (and not nicotinic) agonists (Kasa and Pakaski, 1987).

Ashkenazi et al. (1989) investigated the effect of carbachol on the DNA synthesis of cultured rat astrocytes. These cells were GFAP-positive and had a protoplasmic morphology. Carbachol stimulated the rate of DNA synthesis in the astrocytes and atropine inhibited this effect, indicating a muscarinic receptor involvement. Oxotromorine is a muscarinic agonist that inhibits adenylyl cyclase but does not stimulate phosphoinositol hydrolysis. This agonist has no effect on astrocytic DNA synthesis. Ashkenazi et al. (1989) used cell lines that expressed muscarinic receptor subtypes that if activated stimulated phosphoinositol hydrolysis. These cell lines showed a stimulation of their DNA synthesis after agonist stimulation. Since it was shown that astrocytes express M1 subtype receptors (Murphy et al., 1986), which stimulate phosphoinositol hydrolysis, it is very likely that the effect of cholinergic agonists on astrocytic DNA synthesis is mediated by these M1 receptors. It is likely that these receptors are involved in astrocytic cell growth during development, which happens between day E14 and P15 (the time period the astrocytes were taken from the brain and cultured in the experiments by Ashkenazi et al., 1989). Acetylcholine could have a function as a signal substance to coordinate the development of astrocytes with the activity of cholinergic neurons.

SEROTONIN

Glial Cells of the Leech

Serotonin is one of the most important neurotransmitters in the leech central nervous system (Lent and Dickinson, 1984). It can easily interchange between central nervous system and blood because the central nervous system of the leech is directly located in the ventral blood sinus and there is no blood-brain barrier. Thus, it is thought that serotonin, released by central neurons, circulates in the blood playing a hormonal or modulator role and has an important function in regulating behavioral states such as arousal (Willard, 1981). Therefore glial cells could very well be exposed to different amounts of serotonin, depending on the overall state of activity in the animal.

It was found that serotonin hyperpolarized leech neuropile glial cells by -7 mV and decreased the input resistance to one-third of its original value (Walz and Schlue, 1982). The response had a reversal potential that behaved like a Nernst potential for K^+ when external K^+ concentrations were changed. Exposure to serotonin led to Cl^- release from the cells (Ballanyi and Schlue, 1990) as well as a shrinkage of the cell volume by 14% (Ballanyi et al., 1990). This indicates that the serotonin-induced changes are due to action on a K^+ conductance, which causes passive KCl and water efflux. Since during neuronal activity these neuropile glial cells take up KCl and swell (Wuttke, 1990), one might speculate that this serotonin-induced KCl release is a possible release process for this transiently stored K^+ (Walz, 1982).

There are indications that serotonin not only modulates ion conductances in leech glia, but also has a metabolic effect. Seal and Pentreath (1985) found that serotonin modulates the glycogen content in the

glial cells of leech segmental ganglia. Such an action is similar to the one in mammalian astrocytes.

Mammalian Astrocytes

Receptor Types and Distribution. Fillion et al. (1980) used a bulk-isolated preparation from horse striatum and reported two binding sites for serotonin. Using kainic acid to destroy postsynaptic binding sites, they found that only one of them was removed. This indicates that one of the binding sites is not located postsynaptically. Since there are no presynaptic binding sites (Whitaker and Deakin, 1981), a glial localization for this serotonin binding site was proposed; It was the lower affinity site (K_D = 10 nM), and the pharmacological profile did not fit with any known subtype receptor. However, the study confirmed that glial cells *in vivo* possess serotonin-binding sites.

Hertz et al. (1979) was the first to report a serotonin-binding site for cultured astrocytes. Whitaker-Azmitia and Azmitia (1986) found that treatment of the cultures with dbcAMP reduced the number of binding sites to one-third. There is a controversy about the subtypes of serotonin receptors found on cultured astrocytes. Much of the earlier work on cultured astrocytes (Hertz et al., 1979; Tardy et al., 1982; Whitaker-Azmitia and Azmitia, 1989) did not use specific antagonists against receptor subtypes. Many studies used methylsergide, which is a 5-HT_1, and a 5-HT_2 receptor antagonist. Thus, in the earlier work, it was proposed that a 5-HT_{1A} and/or 5-HT_{1B} receptor exists in cultured astrocytes. However, Whitaker-Azmitia and Azmitia (1989) and Whitaker-Azmitia et al. (1990) reported the presence of 5-HT_{1A} receptors on astrocytic cultures from various brain regions. This report contradicts the results by other groups especially those by Deecher et al. (1993). However, it should be pointed out that the case for the existence of 5-HT_{1A} receptors is indirect via a functional assay (see below), and not established by binding assays. There was also early evidence for a 5-HT_2 receptor, since ketanserin (a 5-HT_2 antagonist) inhibited serotonin effects (Hosli and Hösli, 1987; Hansson et al., 1987, 1990; Nilsson et al., 1991a; Deecher et al., 1993). In a detailed study by Deecher et al. (1993) using radioligand binding and mRNA analysis, no evidence was found for 5-HT_{1A} or 5-HT_{1B} receptors. They confirmed the occurrence of 5-HT_2 receptors. This occurrence of 5-HT_2 receptors was found for all brain regions studied. Other evidence for the existence of 5-HT_2 receptors, and not 5-HT_1 receptors, comes from studies of second-messenger systems (see below). 5-HT receptors are connected to the adenylyl cyclase, whereas 5-HT_2 receptors are coupled to phosphoinositol hydrolysis. Available evidence clearly connects 5-HT effects with phosphotidylinositol hydrolysis and not with direct effects on adenylyl cyclase (to be discussed in the next section). Thus the available data are heavily in favor of the existence of 5-HT_2 receptors. One cannot exclude the possibility that some heterogeneity exists in the culture systems used by different investigators. This may lead to 5-HT_{1A} or 5-HT_{1B} expression in some of them.

Activation of Signal Transduction Systems. Work by Fillion et al. (1980) suggested a link between the suspected glial binding site and adenylyl cyclase activity. However, neither work on cultured astrocytes nor C6 glioma cells found any evidence for a direct 5-HT receptor link to adenylyl cyclase (Ebersolt et al., 1981; Chneiweiss et al., 1984; Hansson et al., 1987, 1990). Hansson et al. (1990) found that isoproterenol-stimulated accumulation of cAMP was potentiated by a 5-HT_2 receptor activation in cultured astrocytes. Phosphorylation by protein kinase C of the catalytic subunit of adenylyl cyclase is seen as a possible mechanism.

The investigations on cultured astrocytes (Hansson et al., 1987; Murphy and Welk, 1990) and C6 glioma cells (Ananth et al., 1987) all point to an induction of phosphatidylinositol hydrolysis by a 5-HT_2 receptor activation as the mechanism of action for serotonin. This was the mechanism in cultures from regions as diverse as cerebral cortex, striatum, hippocampus, and brainstem. This hydrolysis of phosphatidylinositol should lead, via IP_3, to Ca^{2+} mobilization from internal stores. Several authors measured such a Ca^{2+} mobilization in astrocytes during serotonin exposure (McCarthy and Salm, 1991; Nilsson et al., 1991). It was found for type 2-like (stellate) astrocytes in culture (Dave et al., 1991; Inagaki et al., 1991), although not all cells responded. Glioma cells show a 5-HT_2-mediated Ca^{2+} mobilization (Reiser et al., 1989). The mobilization in the astrocytes was shown to be 5-HT_2 mediated as well (Nilsson et al., 1991b). One study provided evidence that the 5-HT_2 mediated Ca^{2+} increase was not only due to Ca^{2+} mobilization, but that there was also a Ca^{2+} influx component (Nilsson et al., 1991). The serotonin effect on the internal Ca^{2+} stores was seen as the mechanism responsible for the serotonin-induced inhibition of the purinergic receptor-mediated arachidonic acid release (Murphy and Welk, 1990).

Thus, at least from the point of view of cell culture studies, there can be little doubt that there is 5-HT_2-mediated hydrolysis of phosphatidylinositol accompanied by Ca^{2+} mobilization. Direct effects on the adenylyl cyclase activity have been ruled out. What

is lacking is confirmation of these results from *in situ* systems. At least one study confirmed a 5-HT_2-mediated phosphatidylinositol hydrolysis in brain tissue (Conn and Sanders-Bush, 1985).

Electrophysiological Effects. In explant cultures from striatum, brainstem, and spinal cord Hösli and Hösli (1987) were able to show that 64% of the astrocytes hyperpolarized (1–8 mV), 13% depolarized, and 23% showed no change in the membrane potential in response to serotonin application. Since ketanserin reversibly antagonized the hyperpolarizations, a 5-HT_2-mediated mechanism appeared likely. This study is contradicted by another, which did not find any electrical changes in cultured astrocytes when serotonin was applied (Kettenmann et al., 1984).

Rat glioma cell lines have a lower membrane potential than astrocytes due to a higher relative Cl^- conductance (Picker et al., 1981; Wolpaw and Martin, 1984). These cells react to serotonin exposure with about −20 mV hyperpolarization and with decreases in their input resistance (Ogura and Amano, 1984). There was a clear desensitization and the reversal potential was close to the K^+ equilibrium potential. A low concentration of quinidine, a Ca^{2+}-dependent K^+ channel blocker, inhibited the response. Further it was found that there was an intracellular Ca^{2+} mobilization (Sugino et al., 1984; Ogura et al., 1986; Reiser et al., 1989). The receptor activation mediates a stimulation of phospholipase C, leading among other products to IP_3, a mediator of Ca^{2+} mobilization (Ananth et al., 1987). Thus for these glioma cell lines the following sequence of events emerges:

1. A 5-HT_2 receptor is activated.
2. Increased phospholipase C activity leads to IP_3 production.
3. IP_3 mediates Ca^{2+} mobilization.
4. Increased cytosolic Ca^{2+} concentrations opens Ca^{2+}-dependent K^+ channels.
5. The increased K^+ conductance causes a hyperpolarization due to the low relative K^+ permeability during resting conditions.

Serotonin Receptors and Growth and Development. There is evidence that serotonin is used as differentiation signal for some embryonic neurons (Lauder et al., 1982). This may involve mediation by glial-derived factors (Whitaker-Azmitia et al., 1990). Thus, when astrocytic cultures from brainstem or cortex are treated with 5-HT_{1A} agonists, they release one or several factors, that when applied to developing serotonergic neurons enhance their development (Whitaker-Azmitia and Azmitia, 1989). Development was measured as an increase in serotonin uptake. Furthermore, it was shown with a specific antibody that at least one of the factors released by astrocytes is the protein S-100β (Whitaker-Azmitia et al., 1990). S-100β stimulates both the differentiation and survival of neurons and the proliferation of astrocytes themselves (Azmitia et al., 1992). There is also some *in vivo* evidence for such an interaction. Rats lesioned with 5,7-DHT, a selective toxin for serotonergic neurons produced an 80% increase in S-100β levels, when treated with the 5-HT_{1A} receptor agonist (Azmitia et al., 1990).

A further test of such involvement of 5-HT receptor mediated release of glia-derived factors in neuronal development was undertaken by Liu and Luder (1992). Serotonin exposure of cultured raphe (serotonergic) neurons and mesencephalic dopamine neurons had larger effects on survival, and cell soma size, in neuronal-glial cocultures. There were some region-specific differences that led to the conclusion that raphe and mesencephalic glia may express different capacities for such an interaction with serotonin and subsequent release of factors influencing neuronal development.

Other Effects on Functional Variables. Several other effects of serotonin exposure to astrocytic function have been described, but none was studied in detail. A glial-enriched brain fraction was used to study Na^+/K^+-ATPase activity (Mercado and Hernandez, 1992). The authors postulated that serotonin exposure will lead to an activation of the glial Na^+ pump through the exposure of more enzymatic active sites. The effects are significant but not dramatic: K_m increased from 6.0 to 9.3 mM and V_{max} from 11.2 to 14.5 μmole $P_i \times mg^{-1}$ protein $\times h^{-1}$ when exposed to a relatively high serotonin concentration of 1 mM.

Hertz et al. (1989) demonstrated that serotonin exposure of astrocytes inhibited high K^+-induced glutamate release. Again, the serotonin concentration was high (1 mM). This effect, however, could be important in metabolic interactions. In this context it is of interest that Cambray-Deakin et al. (1988) have shown that serotonin has no effect on the astrocytic glycogen content. However, if the cultures are reserpine-treated and therefore have a higher glycogen content, serotonin decreases this elevated content by 28%. Thus, serotonin was shown to evoke glycogenolysis under certain circumstances, although its potency is far less than, for example, that of norepinephrine.

CONCLUSIONS

A most interesting feature is the ability of invertebrate Schwann cells to synthesize acetylcholine and release it in response to an axonal signal. Moreover, acetylcholine then subsequently acts on glial conductances. These findings, together with those in leech glial cells, suggest that acetylcholine is a controlling signal in K^+ homeostasis in the invertebrate nervous system.

Muscarinic M1 and M2 receptors and their signal transduction systems have been described in the mammalian brain. There are also some reports of nicotinic receptors on glia. The only convincing experimental evidence that glial muscarinic receptors are functionally important pertains to the control of the development of astrocytes.

Serotonin seems to regulate glial ion content and volume in the leech central nervous system, which may implicate its involvement in ion homeostasis. In the mammalian central nervous system electrical effects seem negligible. There is good evidence for the existence of 5-HT_2 receptors in astrocytes and their involvement in phosphatidylinosite hydrolysis and Ca^{2+} mobilization. No clear concept has emerged about the function of this signal system. Several reports show a close relationship between serotonergic receptor-evoked release of glial factors (i.e., S-100β) and their regulatory influence on neurons. In this case, however, a postulated 5-HT_{1A} receptor seems to be responsible.

Most interesting might be a possible interaction between these transmitter receptors and uptake systems. Ballanyi and Schlue (1989) found that the nicotinic receptor-channel complex can assist in uptake (and maybe release) of choline into glia. Deecher et al. (1993) speculated about a possible interaction between 5-HT_2 receptors and serotonin uptake systems in the mammalian brain: Fluoxetine, an inhibitor of serotonin uptake, seems to decrease the number of the glial 5-HT_2 binding sites in the brain (Asakura and Tsukamoto, 1985).

Taken together, the available evidence suggests that crucial interactions between neurons and glial cells occur and that acetylcholine and serotonin are involved, although the evidence is not yet as convincing as in the cases of glutamate and GABA.

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23 Astroglial adrenergic receptors: expression and function

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While glial cells as a distinct component of the central nervous system were described over a hundred years ago (Virchow, 1858), it is surprising how little we know about the role of these cells in brain function. Shortly after the turn of the century, Lugaro (1907) suggested that glia may play a role in neuronal communication. This speculative report raised the possibility that glia may terminate the action of neurochemicals released from neurons. Many years elapsed before investigators were able to develop preparations that could be used to assess the functions of glia. A major difficulty in assessing the role of astrocytes in central nervous system function is the lack of model systems for studying the properties of these cells *in vivo*. It is very clear from research in many laboratories that astroglia exhibit a wide variety of neurotransmitter receptor and uptake systems, ion channels, growth factors, adhesion molecules, and metabolic processes that may be critical for the normal development and function of the central nervous system. To date, however, no astrocytic functions have been proven to be critical for the function of the adult central nervous system. The lack of information on the role of astrocytes in brain function most likely stems from the difficulty of studying these cells *in vivo*. A similar void was present 20 to 25 years ago concerning the properties of astroglial cells *in vitro*. Through a number of technical and conceptual advances, we are now by comparison overwhelmed with the diverse properties of astroglia that have been established by studies of these cells *in vitro*. Whether or not these properties translate into astrocytic functions *in vivo* remains the quest of many neurobiologists.

EARLY EVIDENCE FOR NEUROTRANSMITTER RECEPTORS ON GLIA

Probably the most important breakthrough since the description of glia by Virchow in the late nineteenth century was the development of methods that could be used to study the properties of these cells *in vitro*. These methods appeared in two stages: first, the identification of transformed cell lines that exhibited glial characteristics *in vitro* (Clark and Perkins, 1971; Gilman and Nirenberg, 1971) and second, the preparation of cultures of nontransformed glia from brain tissue (Booher and Sensenbrenner, 1972; McCarthy and de Vellis, 1980). In 1971, two groups (Clark and Perkins, 1971; Gilman and Nirenberg, 1971) reported that cell lines thought to be of glial origin responded to adrenergic receptor agonists with an increase in cyclic AMP. These studies surprised many neurobiologists who had assumed that neurotransmitter receptors in the central nervous system would be restricted to neuronal elements. One possible explanation for these findings was that the glial cell lines were derived from progenitor cells that exhibit the characteristics of both neurons and glia. In 1972, however, Gilman and Schrier demonstrated that cultures prepared from fetal brain also responded to adrenergic receptor agonists with an increase in cyclic AMP levels and conditions that promoted nonneuronal cell survival led to a greater degree of stimulation. These seminal studies strongly suggested that glial cells had the potential to respond to neurotransmitters released from neurons. Many neurobiologists held to the view that these findings reflected the responses of nonglial cells contained within the population of brain cells. As these studies were being reported, other investigators were developing methods that could be used to prepare purified cultures of astroglia (McCarthy and de Vellis, 1980). These methods and the growing availability of astroglial-specific immunological markers (Bignami et al., 1972; Norenberg and Martinez-Hernandez, 1979) opened the door for an ever-increasing number of neurobiologists to explore the diverse properties of these cells.

During the late 1970s and early 1980s a large number of investigators assessed the ability of astroglial cells to respond to different neuroligands with an increase in cyclic AMP (van Calker and Hamprecht, 1981; van Calker et al., 1978; McCarthy and de Vellis, 1978, 1979; Ebersolt et al., 1981).

The results of these studies demonstrated that essentially all receptors known to affect cyclic nucleotide levels affected this second-messenger system in astroglia. Furthermore, the finding that the stimulation of cyclic AMP levels by one ligand could be modulated by an alternate ligand (van Calker et al., 1978; McCarthy and de Vellis, 1978, 1979; Evans et al., 1984) suggested that individual astroglia exhibited multiple neuroligand receptor systems. Through the 1980s and early 1990s investigators continued to assess the expression of receptor systems either by measuring alternate second-messenger systems (Pearce et al., 1985; Pearce et al., 1986; M. Enkvist et al., 1989a, 1989b; Salm et al., 1990a), directly labeling receptors with isotopically labeled ligands (McCarthy and Harden, 1981; Harden and McCarthy, 1982; Torrens et al., 1986; Yeung et al., 1991) or electrophysiological methods (Walz, 1989; Berger et al., 1992; Bowman et al., 1992; Gimpl et al., 1992; Magoski and Walz, 1992). As these studies continued it became clear that the issue was not which neuroligand receptors were present, but rather, which receptors were missing from astroglia. Currently, there appears to be good agreement that astroglia lack the NMDA (*N*-methyl-D-aspartate) excitatory amino acid receptor *in vitro*. However, recent studies suggest that astrocytes *in vivo* may exhibit a distinct form of the NMDA receptor (Müller et al., 1993).

ASTROGLIA ARE HETEROGENEOUS WITH RESPECT TO THEIR EXPRESSION OF NEUROLIGAND SIGNAL TRANSDUCTION SYSTEMS

Assuming that astroglia exhibit receptors *in vivo* to respond to locally released neurotransmitters, the repertoire of receptors exhibited by these cells should vary within and between brain regions. Initial attempts to test this hypothesis compared the ability of different neuroligands to affect second messengers in populations of astroglia prepared from different brain regions (Cholewinski, and Wilkin, 1988; Wilkin et al., 1990). The results of these studies supported the view that certain agonists were more effective in one brain region than another (Cholewinski, and Wilkin, 1988; Wilkin et al., 1990); however, there were few examples of qualitative differences in the responsiveness of astroglia prepared from different brain regions. To examine the question of receptor heterogeneity in greater detail, methods were developed that enabled the quantitative measurement of the number of neuroligand binding sites on individual immunocytochemically defined cells *in vitro*. Methods were developed to quantify both alpha- and beta-adrenergic receptors using iodinated receptor ligands (Royds et al., 1982; Lerea and McCarthy, 1989). The results of experiments using the beta-adrenergic receptor (β-AR) selective ligand, cyanopindolol, indicate that all type 1-like astroglia (Raff, 1989) examined exhibit β-ARs (Burgess et al., 1985; Burgess and McCarthy, 1985). To date, astroglia isolated from cerebral cortex, cerebellum, hippocampus, and spinal cord have been examined in these studies. In contrast, type 2-like astroglia do not appear to exhibit β-ARs (Burgess and McCarthy, 1985). As type 1- and type 2-like astroglia appear to represent separate glial lineages (Raff, 1989), these findings suggest that there is little heterogeneity in the expression of β-ARs within either lineage of these two groups of glial fibrillary acidic protein (GFAP) positive cells. Furthermore, the ubiquitous expression of β-AR by type 1-like astroglia suggests that whatever functions are influenced by this receptor system, they are common throughout the brain. Neither neurons nor oligodendroglia appear to exhibit a significant number of β-AR binding sites *in vitro* (Burgess et al., 1985).

The expression of alpha$_1$-adrenergic receptors (α_1-ARs) by neural cells was measured using the receptor ligand [^{125}I]HEAT (Lerea and McCarthy, 1989). The results of these studies suggest that both type 1- and type 2-like astroglia are heterogeneous with respect to their expression of this adrenergic receptor system (Lerea and McCarthy, 1989). Interestingly, type 1-like astroglia exhibited a very large range in the density of α_1-ARs expressed. Within a single microscopic field, astroglia were observed to exhibit densities ranging from zero to several thousand binding sites per thousand square micrometers. Approximately 70% of type 1- and type 2-like astroglia exhibit α_1-AR binding sites above nonspecific levels (Lerea and McCarthy, 1989). No evidence for receptor clustering has been observed for either β-ARs or α_1-ARs on astroglia (Lerea and McCarthy, 1989; Burgess et al., 1985). The finding that astroglia vary both qualitatively and quantitatively in the expression of β- and α_1-ARs suggests that these receptor systems are not expressed in response to *in vitro* culture conditions.

It is likely that full astroglial differentiation requires association with neurons. Therefore, experiments were designed to examine the effect of neurons on the expression of α_1-ARs by astroglia. In these experiments, cocultures of neurons and astroglia were prepared from hippocampus and grown under conditions that resulted in astroglia growing both in direct apposition to neurons and away from neurons (Lerea and McCarthy, 1990). These experi-

ments revealed two interesting findings. First, astroglia in direct contact with hippocampal neurons often assumed an elongated morphology that closely resembled that of neurons (Lerea and McCarthy, 1990). These elongated astroglia did not resemble type 2-like astroglia in their morphology. Furthermore, direct contact between neurons and astroglia was required to induce the morphological change in astroglia, as those separated from neurons by only a few micrometers failed to become elongated. Second, approximately 70% of astroglia growing in direct contact with neurons continued to exhibit α_1-AR binding sites. Overall, the results of these studies indicate that astroglia are heterogeneous in their expression of binding sites for α_1-AR ligands and that these cells continue to express α_1-ARs when grown in intimate association with neurons. These findings support the premise that α_1-ARs are distributed similarly on astrocytes *in vivo*.

As evidence accumulated that astroglia exhibit a large number of receptor systems, an interesting question emerged: Can pharmacologically distinct subsets of these cells be identified? To address this issue, the influence of neuroligands on calcium mobilization in groups of individual astroglia in nonconfluent cultures was examined. In these experiments, astroglia were loaded with the calcium indicator dye fura-2, and the response of individual cells was monitored with a video-based imaging system (Salm and McCarthy, 1990b; McCarthy and Salm, 1991). Through these experiments it became clear that astroglia exhibit a number of different receptor systems linked to calcium regulation and that marked heterogeneity exists among the cells with respect to their ability to respond to neuroligands with a rise in intracellular calcium (McCarthy and Salm, 1991). Neuroligands effective in increasing calcium levels in astroglia include those that stimulate alpha$_1$- and alpha$_2$-adrenergic, -histaminergic, -muscarinic, -P_{2Y} purinergic, -glutaminergic, and -serotonergic receptors (McCarthy and Salm, 1991). No evidence for the grouping of subsets of these receptor systems among astroglia was observed. In general, the efficacy of different neuroligands was similar with respect to their ability to increase calcium levels in astroglia. However, the percentage of astroglia responding to the different neuroligands with a rise in intracellular calcium levels varied markedly (McCarthy and Salm, 1991). Most of the neuroligands examined appeared to increase intracellular calcium levels both through the release of internal stores and the opening of plasma membrane calcium channels (Salm and McCarthy, 1990b).

The observation that very few examples exist of cells switching their receptor phenotype suggested that the pharmacological heterogeneity of astroglia was likely to be "locked" and to reflect distinct and stable subsets of cells. Two approaches were used to test this hypothesis. First, astroglia were plated at cloning density, allowed to develop into clones containing from 2 to 16 cells, and then assessed for their ability to respond to neuroligands with a rise in intracellular calcium levels. In marked contrast to expectations, the individual cells of an astroglial clone varied in their ability to respond to neuroligands with a rise in Ca_i^{2+}. For example, immediately following the division of a parent cell, the two daughter cells were qualitatively distinct with respect to their ability to respond to neuroligands with a rise in Ca_i^{2+} (Shao and McCarthy, 1993). The design of these experiments left open the possibility that astroglia of a single clone would eventually express the same set of receptor systems if maintained *in vitro* for an extended period of time. To address this question, astroglia were transfected with a retrovirus that enabled selection for transfected clones via antibiotic resistance. The results of these experiments indicated that astroglia within a single clone remain pharmacologically distinct after 4 weeks *in vitro* (Shao and McCarthy, 1993). These findings indicate that intrinsic clocks persist in astroglia *in vitro,* which lead to the diversification of these cells. These experiments did not directly address the question of whether the receptor signaling systems of individual astroglia change over time. This question was examined by monitoring the calcium responsiveness of the same individual astroglia within clones at intervals of 1 to 3 days. The results of these experiments indicated that the ability of individual astroglia to respond to neuroligands changes over time *in vitro.* In general, astroglia develop sensitivity to α_1-AR agonists during the first week *in vitro* (Shao and McCarthy, 1993). However, it is evident that cells may either develop or lose their ability to respond to a neuroligand over the course of only a few days *in vitro.*

It became apparent during the course of these experiments that astroglia tended to lose their sensitivity to carbachol (a muscarinic cholinergic agonist) over several days of testing. The initial assumption was that the loss in sensitivity to carbachol reflected a developmental process inherent in astroglia. However, a similar percentage of previously untested astroglia responded to carbachol with a rise in Ca_i^{2+} after 1, 2, or 3 weeks *in vitro* (Shao and McCarthy, 1993). It is now apparent that brief stimulation of astroglia with carbachol (5 minutes per day for 3 days; 1 mM) triggers the downregulation of muscarinic cholinergic signal transduction (Shao and McCarthy, 1993). These findings suggest that brief exposure to calcium

mobilizing ligands selectively affects astroglial muscarinic cholinergic receptor expression (Shao and McCarthy, 1993). To date, similar findings with other cells that exhibit muscarinic receptors linked to calcium mobilization have not been observed. Collectively, these findings indicate that both intrinsic and extrinsic signals affect the expression of astroglial signal transduction systems.

ASTROGLIA EXHIBIT AN ALL-OR-NOTHING CALCIUM RESPONSE TO ALPHA$_1$-ADRENERGIC RECEPTOR STIMULATION

Previous studies indicated that approximately 70% of type 1-like astroglia exhibit α_1-AR binding sites (Lerea and McCarthy, 1989). A surprising finding in those studies was the wide range in the density of α_1-AR binding sites (a few over background to several thousand per 1000 μm^2) exhibited by astroglia. Interestingly, subsequent experiments found that a similar percentage of the cells responded to α_1-AR selective ligands with an increase in Ca_i^{2+}. A series of experiments were designed to examine the quantitative relationship between the density of α_1-AR binding sites and the ability of astroglia to respond to α_1-AR selective ligands with a rise in Ca_i^{2+}. In these experiments, individual astroglia were first analyzed for their Ca^{2+} sensitivity to phenylephrine (an α_1-AR-selective ligand) and then their expression of α_1-AR binding sites and GFAP were determined. Surprisingly, little correlation was found between the density of α_1-ARs and the magnitude of the rise in Ca_i^{2+} in astroglia following treatment with phenylephrine (Figures 23-1 and 23-2). Dose-effect experiments supported the poor correlation between the number of α_1-ARs occupied and the magnitude of the Ca_i^{2+} response in these cells (Figure 23-3). Together, the results of these studies suggest that astroglia need only a few α_1-AR binding sites occupied to elicit a full Ca^{2+} response. In contrast to the magnitude of the Ca^{2+} response, both the probability of responding and the latency to response (the time between drug addition and initial rise in Ca_i^{2+}) correlated strongly with the density of α_1-ARs exhibited by astroglia and the dose of phenylephrine applied (Figures 23-4 and 23-5). These findings suggest that activation of α_1-ARs results in the accumulation of a second messenger (presumably IP_3), which must reach a threshold concentration before eliciting what is then an all-or-nothing Ca^{2+} response. Further-

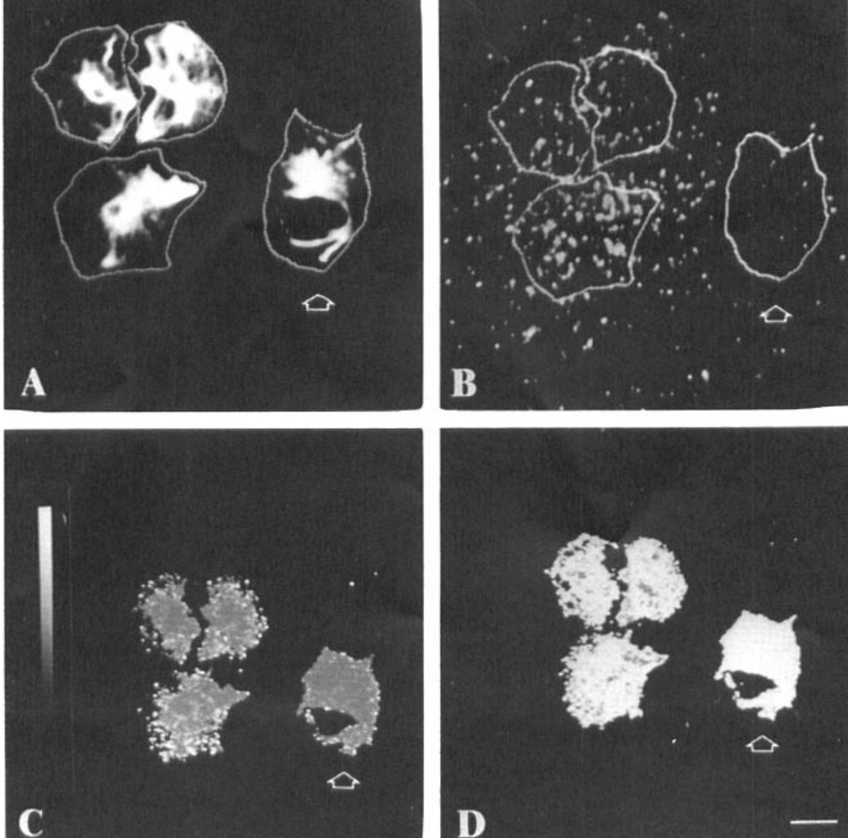

FIG. 23-1. Ca^{2+} responses in astroglia with different α_1-AR densities. Cells in primary culture were loaded with fura-2 and tested for Ca^{2+} responses, and subsequently processed for combined GFAP immunocytochemistry and α_1-AR receptor autoradiography. Digitized images show 4 GFAP positive cells (A) with various grain densities (B). Following addition of 10 μM phenylephrine, all cells increased $[Ca^{2+}]_i$ from the basal (C) to the maximal level within 5 seconds (D). Bar = 10 μm.

FIG. 23-2. Ca^{2+} responses in individual astroglial cells as a function of α_1-AR density. Each dot represents a cell that was exposed to 1 μM phenylephrine and subsequently processed for the α_1-AR binding autoradiography. A total of 107 cells that had an α_1-AR density above the nonspecific level are presented. The *y*-axis represents the increase in Ca_i^{2+} levels above basal levels (peak response − basal level).

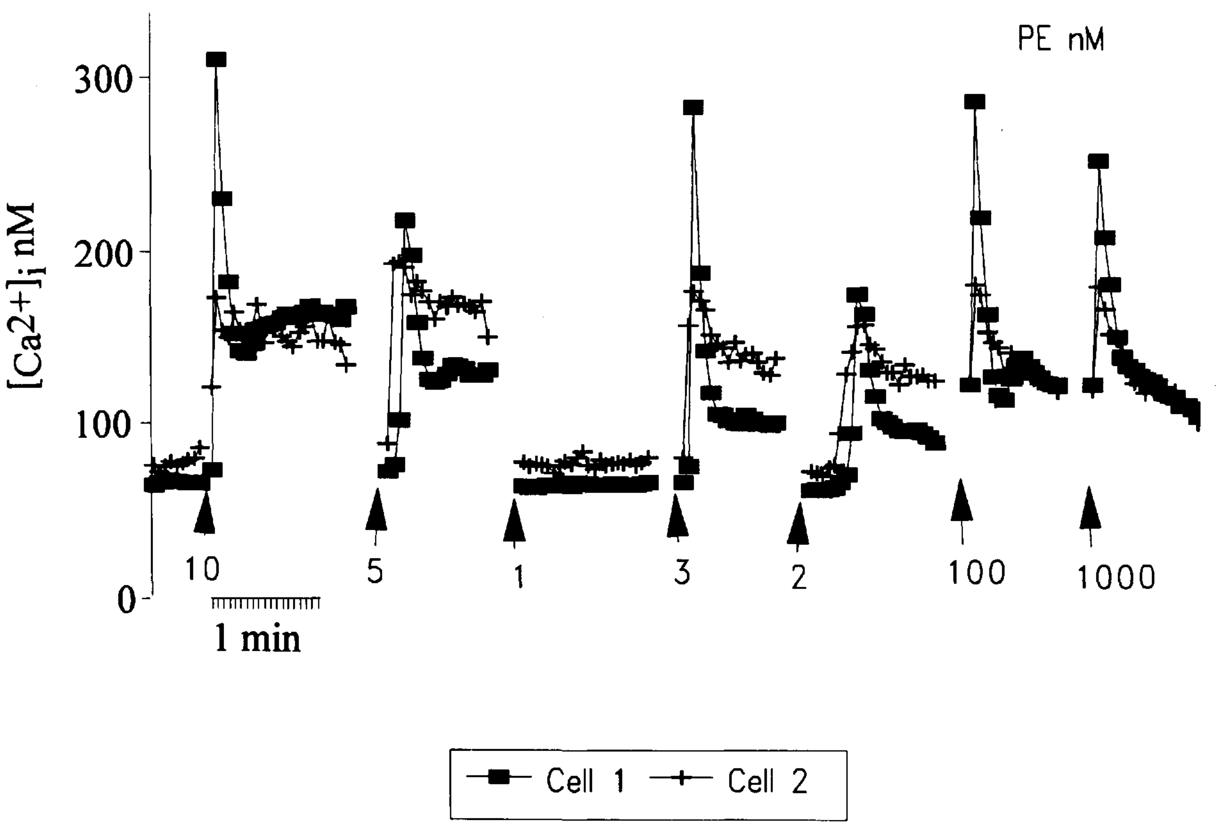

FIG. 23-3. Dose-response of astroglial cells to various concentrations of phenylephrine. The two cells were treated with phenylephrine with a change of the full-bath solution (*arrows*). Between each treatment, the cells were rinsed with ligand-free HBSS 3 times, and incubated for at least 5 minutes. The sampling rate is 3 seconds per measurement.

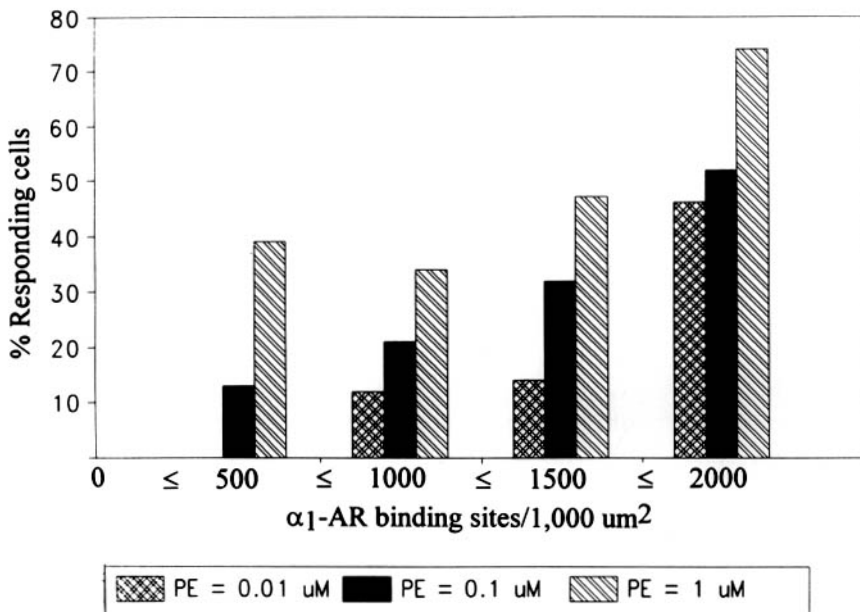

FIG. 23-4. Relative frequency of astroglial responsiveness to various doses of phenylephrine in relation to the α_1-AR density. Cells were treated with 10, 100, and 1000 nM phenylephrine successively, and subsequently processed for α_1-AR binding autoradiography. Cells were grouped according to their α_1-AR density (bin width = 500 binding sites per 1000 μm^2, n = 36).

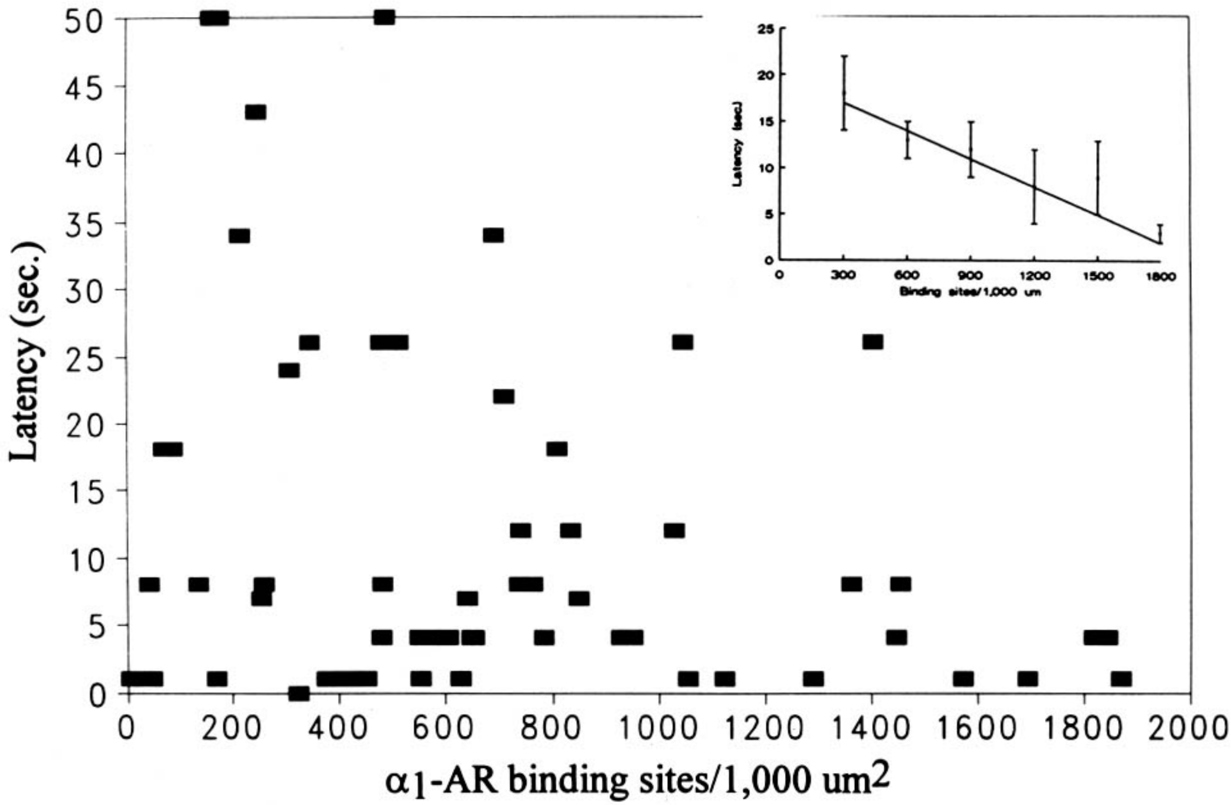

FIG. 23-5. Relationship between latencies of Ca^{2+} responses and α_1-AR densities on individual astroglial cells. Each dot represents the time between the addition of the ligand and the onset of the response plotted against the α_1-AR densities of 51 cells that responded to 10 μM phenylephrine. Inset: The data are MEAN ± SEM of the average time of cells grouped according to their α_1-AR density with the bin width of 300 binding sites per 1000 μm^2. The line is derived from linear regression with a correlation coefficient of 0.95.

more, the observation that the probability of responding is correlated with the density of α_1-AR binding sites suggests that the rate of second-messenger accumulation is important in determining whether or not cells respond to α_1-AR stimulation with a rise in Ca_i^{2+}. One possibility is that IP_3 must accumulate to a given concentration to elicit a Ca_i^{2+} response. When only a few α_1-ARs are available for stimulating phospholipase C, cells desensitize prior to accumulating sufficient IP_3 for Ca^{2+} release. The finding that astroglia exhibit an all-or-nothing response suggests that feed-forward processes are involved in the regulation of Ca_i^{2+} in these cells. The observation that astroglia with low α_1-AR densities occasionally respond to phenylephrine with a rise in Ca_i^{2+} suggests that other processes are also important in causing Ca^{2+} release from internal stores. One possible explanation for this finding would be spatial coupling of the different components (α_1-ARs, G proteins, PLC, IP_3-sensitive stores) required for Ca^{2+} release. The observation that ligands tend to initially stimulate an increase in Ca_i^{2+} from a given point in astroglia support the view that one or more of the components of the response system are spatially restricted.

Collectively, the above findings suggest that the regulation of Ca^{2+} responses in astroglia quite closely resembles the regulation of action potentials in neurons. That is, ligands induce an all-or-nothing Ca^{2+} response, which initiates from a given point in the cells and then propagates through the cell in a regenerative manner. Furthermore, the effect of different ligands that increase IP_3 levels is integrated such that cells that would not respond to either ligand alone respond to the combination with a full Ca^{2+} response. Finally, like the generation of action potentials in neurons, both the probability of responding and the latency to response are correlated with the number of occupied receptors in astroglia. We envision that *in vivo* astrocytes integrate the stimulation of multiple ligands to reach a threshold level of IP_3, which then leads to a burst of Ca_i^{2+}, which then moves through individual astroglia and the astrocytic syncytium in a regenerative manner. The role that these Ca_i^{2+} waves play in brain physiology remains an important question in glial biology (see Chapter 17).

ASTROCYTES *IN VIVO* EXHIBIT ALPHA$_1$- AND BETA-ADRENERGIC RECEPTORS

While abundant evidence exists that astroglia exhibit a large number of receptor systems *in vitro*, it has been much more difficult to establish that mature astrocytes *in vivo* exhibit similar receptor systems. The lack of data supporting the presence of receptors on astrocytes *in vivo* stems in large part from the difficulty in preparing purified preparations of astrocytes from mature brain tissue. However, two different approaches have been used to study the expression of adrenergic receptors by mature astrocytes and both suggest that astrocytes exhibit these receptors (Salm and McCarthy, 1989; Aoki and Pickel, 1992; Shao and Sutin, 1992). The β-AR selective ligand [^{125}I]cyanopindolol and dry-mount autoradiography were used to examine the expression of β-ARs by individual fibrous and protoplasmic astrocytes isolated from adult rat brain. The results of these studies indicate that all astrocytes isolated and identified by their GFAP staining exhibit β-ARs (Salm and McCarthy, 1989). Similar findings were reported by Shao and Sutin (1992) for glial cells of the trigeminal motor nucleus. Other investigators have used antibodies to β-ARs to demonstrate that glial elements exhibit this receptor subtype (Aoki and Pickel, 1992). Interestingly, an antibody directed against the β_2-AR subtype was used in these experiments. Results from these studies suggest that β_2-ARs are not uniformly expressed by astrocytes *in vivo*. Furthermore, earlier studies suggested that astroglia in culture exhibit the β_1-AR subtype. While additional experiments with alternate receptor subtype specific antibodies are required, present data support the view that the subtype of β-ARs expressed by astrocytes may change during development. Regardless of the receptor subtype, it is clear that astrocytes *in vivo* exhibit beta-adrenergic receptors and are a target for norepinephrine released in brain. A similar conclusion can be drawn from studies using freshly isolated mature astrocytes and [^{125}I]HEAT to label α_1-ARs (Shao and Sutin, 1992). In these experiments Shao and Sutin (1992) found that a subpopulation of mature astrocytes isolated from brainstem exhibited α_1-ARs.

REGULATION OF GAP JUNCTIONS IN ASTROGLIA BY LIGANDS LINKED TO THE ACTIVATION OF PROTEIN KINASE C

Numerous studies indicate that astroglia *in vitro* (Fischer and Kettenmann, 1985; Sontheimer et al., 1990; Dermietzel et al., 1991) and astrocytes *in vivo* (Connors et al., 1984) are often connected to one another via gap junctions. In astrocytes, gap junctions are composed of 12 units of connexin43, 6 contributed from each of the coupled astrocytes (Yamamoto et al., 1990; Bennett et al., 1991; see Chapter 19, this volume). Connexin43 is also the gap junction protein responsible for the formation of gap junctions in

heart. A number of reports indicate that waves of Ca^{2+} move through an astroglial syncytium via gap junctions (Cornell-Bell et al., 1990; Charles et al., 1991). Similar Ca^{2+} waves have been reported in astrocytes in brain slice cultures (Dani et al., 1992). These findings suggest that an extraneuronal communication network consisting of coupled astrocytes and Ca^{2+} waves may exist in brain. There seems to be two broad views of how this network might work. First, astrocytic signaling over distances via Ca^{2+} waves could be directional, with gap junctions open along certain paths and closed along others. Alternatively, astrocytes may be coupled in a restricted region such that a response in an individual astrocyte results in the activation of a group of astrocytes but without a directional component. In either case, regulation of the opening and closing of astrocytic gap junctions would influence the degree of astrocytic activation. Other investigators have reported that second-messenger systems effectors (e.g., protein kinase C (PKC), protein kinase A (PKA), Ca^{2+}) regulate the opening and closing of gap junctions (Bennett et al., 1991). A series of experiments were completed to examine the effect of treatments that stimulate PKC activity on gap junction communication between astroglia. Two different approaches were used to examine gap junction communication between astroglia. First, the number of cells coupled was determined following injection of a single cell with lucifer yellow. Second, the distance traveled by calcium waves through astroglia following the stimulation of a single cell with a mechanical probe was measured. The results of these experiments indicate that treatments that stimulate PKC activity decrease gap junction communication between astroglia (Enkvist and McCarthy, 1992). When PKC was downregulated by a 24-hour pretreatment with phorbol 12-myristate-13-acetate (PMA), the reduction in gap junction communication caused by brief treatment with either PMA or P_{2Y}-purinergic receptor agonists (which activate PLC in most astroglia) was blocked (Enkvist and McCarthy, 1992). Similar findings have been reported by Giaume et al. (1991). These findings suggest that the coupling of astrocytes *in vivo* may be regulated by neurotransmitters released during neuronal activity. The regulation of gap junction communication between astrocytes may play an important role in events ranging from spatial buffering to the release of trophic factors (see Chapter 19, this volume).

Dye coupling between astroglia was recently reported to be increased by membrane depolarization (Enkvist and McCarthy, 1994). In these experiments astroglia were depolarized by either increasing the concentration of extracellular K^+ or by adding ionophores and the degree of astroglial coupling measured following injections with lucifer yellow. Increasing extracellular K^+ led to a dose-dependent increase in dye coupling between astroglia (Figure 23-6). Gramicidin, a nonspecific cation ionophore that leads to depolarization (Ishii and Volpe, 1992), also increased dye coupling between astroglia (Figure 23-6). In contrast, valinomycin, a K^+ specific ionophore that hyperpolarizes cells, decreased dye coupling between astroglia (Figure 23-6). Finally, treatment with either glutamate, kainate or quisqualate, ligands known to depolarize astroglia (Enkvist et al., 1988), also led to an increase in gap junction communication between astroglia (Figure 23-7). Overall, these findings suggest that the degree of coupling between astrocytes *in vivo* may be regulated by neuronal activity and that specific pathways of extraneuronal communication within the astrocytic syncytium may be important in brain function.

PHYSIOLOGICAL SIGNIFICANCE OF NEUROLIGAND RECEPTORS ON ASTROCYTES

While it is clear that both astroglia *in vitro* and astrocytes *in vivo* exhibit adrenergic receptors, the role that these and other receptor systems play in brain function is less evident. Most studies aimed at elucidating the role of astrocytic receptors in brain function have utilized primary cultures of astroglia. While these *in vitro* preparations provide useful insight into the potential function of astrocytes *in vivo*, it remains uncertain whether properties expressed *in vitro* accurately reflect those expressed *in vivo*. However, it is very likely that the diverse properties characteristic of astroglia *in vitro* are representative of astrocytes *in vivo* at some stage of development.

Early studies with glioma cell lines suggested that glial cells may be important in supporting neurons through the release of neurotrophic factors (Schwartz et al., 1977). It is now clear from the work of many investigators that glial cells synthesize and release a number of different factors that are necessary for neuronal survival and growth (Varon et al., 1987; Ferrara et al., 1988; Houlgatte et al., 1989; Schwartz and Mishler, 1990; Zafra et al., 1992). Neuroactive factors released from astroglia include nerve growth factor, enkephalin peptides, angiotensinogen, taurine, nitric oxide, eicosanoids, S-100, ciliary neurotrophic factor, brain-derived neurotrophic factor, and neurotrophin 3 (Martin, 1992). Additional neuroactive substances have been reported to be present in astroglia and may also be released to affect neuronal activity (Martin, 1992; see Chapters 48 and 49, this volume). These findings strongly sug-

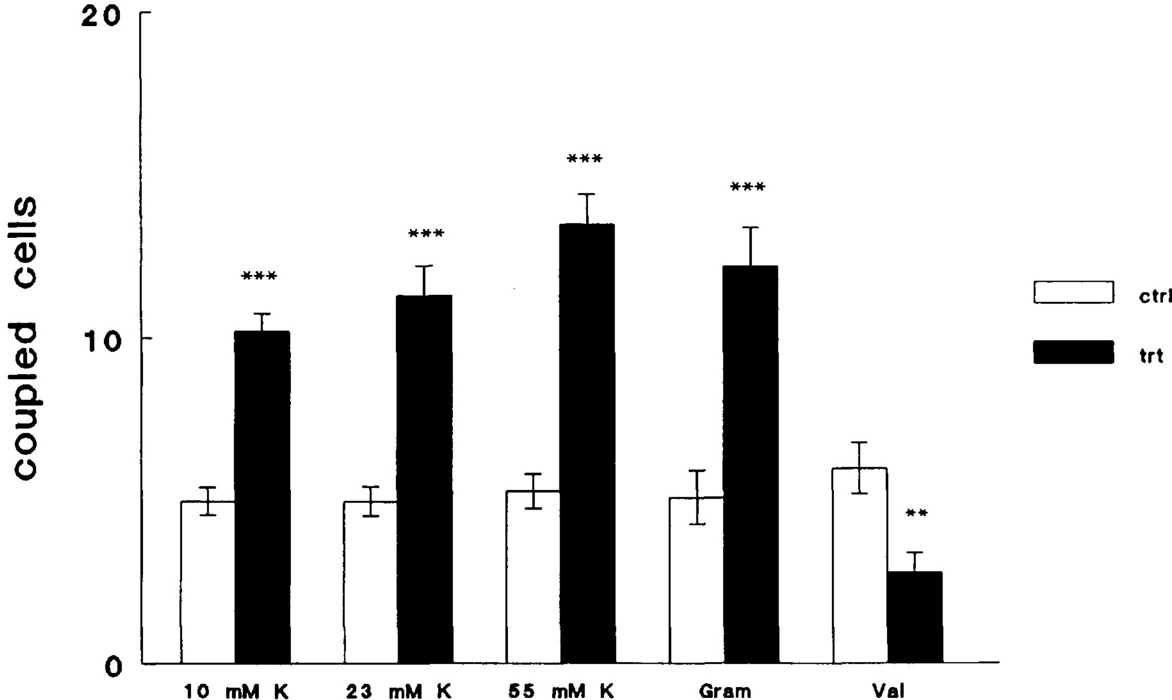

FIG. 23-6. Number of cells coupled to the cell initially injected with lucifer yellow dissolved in buffer containing 65 nM Ca^{2+}. Cells were incubated with the effectors for 10 minutes. Each treatment (*dark bars*) was preceded by injections on the same coverslip under control conditions (*white bars*). The three first treatments represent substitution of extracellular K^+ for Na^+ to 10, 23, and 55 mM K^+. The last two treatments were with ionophores; *Gram*, 2 μM gramicidin; *Val*, 2 μM valinomycin. Data from several coverslips were pooled and is presented ± SEM. *** $p < .001$, ** $p < .005$, Student's *t* test. *n* = 2, 3, 6, 3, 3 coverslips, respectively, with 10 cells injected for each treatment on one coverslip.

gest that astrocytes *in vivo* may influence the growth and differentiation of neurons. It is evident that the release of a number of these different neuroactive substances is controlled by neuroligand receptor activation. For example, work by many investigators make it clear that astroglia contain nerve growth factor (NGF) and that the release of NGF is stimulated by receptor agonists (Schwartz et al., 1977; Furukawa et al., 1987; Houlgatte et al., 1989; Schwartz and Mishler, 1990; Carman-Krzan et al., 1991; Rudge et al., 1992; Zafra et al., 1992). Enkephalin peptides (Melner et al., 1990; Batter et al., 1991), S-100 (Barger and Van Eldik, 1992), and taurine (Shain et al., 1986) also appear to be released by astroglia. As all type 1-like astroglia examined to date exhibit beta-adrenergic receptors, it seems that one role of these receptors may be to increase the release of these neuroactive substances in brain during development. Interestingly, beta-adrenergic receptor stimulation has been reported to open L-type Ca^{2+} channels via the activation of PKA (Bowman and Kimelberg, 1987), whereas stimulation of alpha$_1$-adrenergic receptors results in depolarization of astroglia (MacVicar and Tse, 1988). Overall, it is evident that astroglia exhibit a number of different signal transduction systems, which may influence the synthesis and release of neuroactive factors from astroglia. A major question yet to be resolved is the level to which astrocytes contribute to the release of neurotrophic factors in the adult brain. It remains possible, if not likely, that immature astrocytes contribute to the release of neurotrophic factors during development, while such factors are provided primarily by neurons in the adult brain. Ongoing research in a number of laboratories is aimed at this question.

The idea that astrocytes in brain supply neurons with metabolic nutrients (e.g., glucose) was raised by the early anatomists and is supported by a number of *in vitro* studies (Cummins et al., 1983; Rosenberg and Dichter, 1988; Pfeiffer et al., 1992; Sorg and Magistretti, 1992). In brain, glycogen appears to be located almost exclusively in astrocytes (Peters et al., 1976). Glycogen breakdown in astrocytes is stimulated by agents that increase either cyclic AMP or Ca^{2+} (Cummins et al., 1983; Krutsay, 1986; Sorg and Magistretti, 1991). Furthermore, it has been reported that glycogen phosphorylase (the rate-determining enzyme in glycogenolysis in brain) is restricted in brain to astrocytes (Pfeiffer et al., 1992). These findings

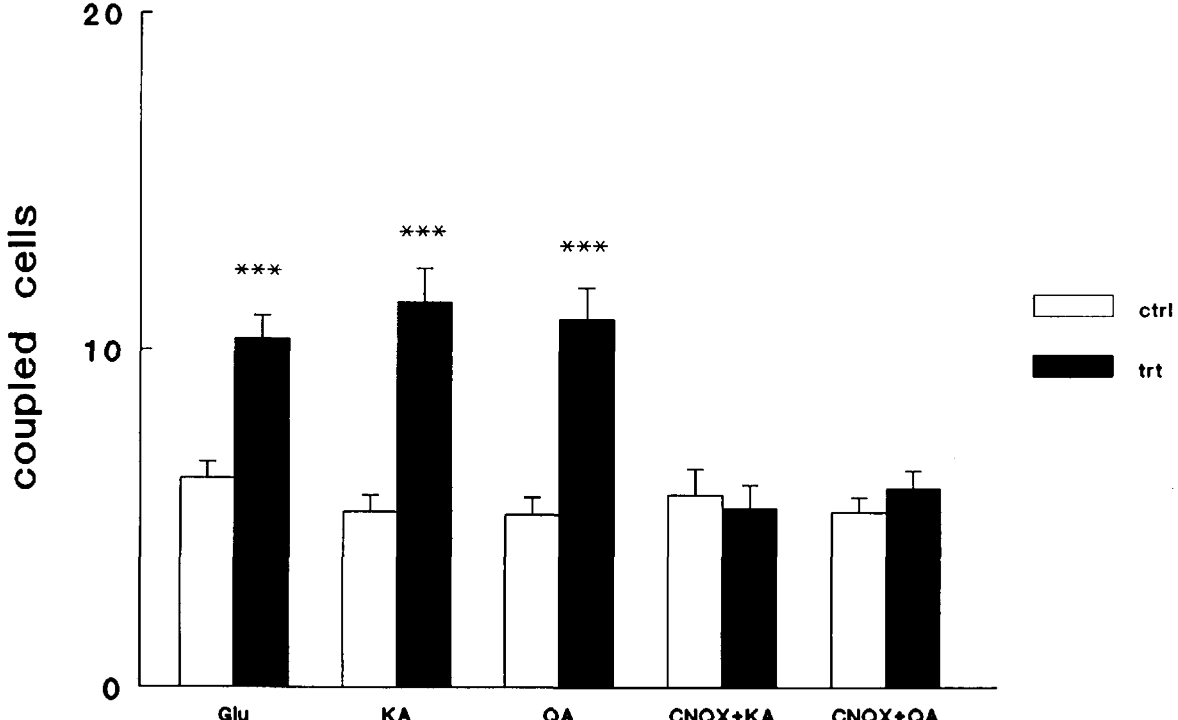

FIG. 23-7. Number of cells coupled to the cell initially injected with lucifer yellow dissolved in buffer containing 65 nM Ca^{2+}. Each treatment (*dark bars*, 10 injections per coverslip) was preceded by injections on the same coverslip in control buffer (*white bars*, 10 injections per coverslip). *Glu*, treatment with 400 μM glutamate for 20 min; *KA*, 400 μM kainate for 20 minutes; *QA*, 400 μM quisqualate for 20 minutes; CNQX+KA, CNQX+QA, treatment with 10 μM CNQX and 400 μM kainate or quisqualate; respectively, for 20 minutes. Data from several coverslips were pooled and is presented ± SEM. *** $p < 0.001$, Student's t test. n = 6, 4, 4, 3, 3 coverslips, respectively.

strongly suggest that in brain, astrocytes may respond to adrenergic receptor stimulation (either alpha- or beta-ARs) with the breakdown of glycogen and release of metabolic substrates to be utilized by neurons.

A number of investigators have reported that neuroligands that increase either PKA or PKC activity affect the morphology of astroglial cells *in vitro*. While the mechanism by which this occurs remains unclear (Pollenz and McCarthy, 1986), increasing the activity of either PKC or PKA converts astroglia from a flat, polygonally shaped cell to a process-bearing cell (Narumi et al., 1978; Mobley et al., 1986; Shain et al., 1987). The morphological conversion of these cells induced by increases in cyclic AMP frequently has been interpreted as reflecting an increase in the differentiation of astroglia. While this may be the case, it is difficult to argue given the absence of markers for mature astrocytes. A different view of the morphological change is that this process reflects the ability of astrocytes to change shape in response to exposure to neuroligands. Changes in astrocytic shape *in vivo* could affect neuronal excitability and synaptic activity by changing the volume of the extracellular space and/or neuronal connections. Changes in astrocytic morphology *in vivo* have been reported by investigators studying glia during lactation (Salm et al., 1985). Additional experiments will be required to determine whether changes in astrocytic morphology *in vivo* are a general property of these cells.

SUMMARY AND FUTURE DIRECTIONS

It is clear that astroglia *in vitro* and astrocytes *in vivo* exhibit adrenergic receptors. The role that these and other astroglial neuroligand receptors play in brain function is less evident. *In vitro* studies indicate that while all astroglia exhibit β-ARs, only a percentage of these cells exhibit α_1-ARs. The fact that astroglia are heterogeneous with respect to their expression of neuroligand receptors *in vitro* indicates that these receptor systems are not expressed in response to *in vitro* growth conditions. In contrast to our expectation, the expression of a given receptor system by astroglia changes over time such that cloned cells grown in an identical chemical and cellular environment become pharmacologically heterogeneous. These findings indicate that intrinsic

clocks persist in astroglia *in vitro* and determine their ability to respond to their local chemical milieu. Even more surprising was our demonstration that brief exposure to ligands that increase Ca_i^{2+} results in the loss of muscarinic cholinergic responses in astroglia. The loss of muscarinic sensitivity appears to be unique in that other cells examined failed to exhibit similar regulation. These findings suggest that astrocytes may undergo qualitative changes in their ability to respond to neuroligands and that these changes may be regulated by neuroligands released at specific times during development. Studies indicate that astroglial cells continue to express neuroligand receptors even when grown in direct contact with neurons and exhibiting a morphology different from astroglia growing a short distance away.

Overall, these findings suggest that the signal transduction systems of astrocytes *in vivo* may change during development in response to both intrinsic and extrinsic signals. By extension, the degree to which the activity of these cells is influenced by neuroligands released from neurons is likely to change during development. We hypothesize that during development immature astrocytes exhibit a large number of neuroligand receptors and that these are necessary to interact with a changing neuronal environment. With brain maturation, the number of neuroligand receptor systems expressed by astrocytes markedly decreases to a number dependent on the heterogeneity of the local neuronal environment. The role that astrocytic receptor systems play in mature brain remains elusive.

Results indicating that astroglia respond to neuroligands with an all-or-none rise in Ca_i^{2+}, which then moves through astroglia in a regenerative manner, are very reminiscent of neuronal action potentials. It seems likely that neuroligand-induced Ca_i^{2+} waves represent an extraneuronal signaling system that may serve to modulate neuronal activity. Modulation could occur through a number of processes, including changes in the ionic composition of the extracellular space, release of substances that would directly affect neuronal excitability, and the physical rearrangement of neuronal synapses.

The amount of information on astroglia that has accumulated during the past two decades is very impressive. However, of the many different properties that have been described using astroglia *in vitro,* few if any are known to be critical to normal brain function in the mature animal. To date, there is not a single example where the perturbation of an astrocytic property leads to a defined deficit in brain function. Indeed, it remains controversial whether astroglia *in vitro* reflect the properties of immature, mature or pathological astrocytes. To establish the importance of astrocytes in brain function, it will be necessary to develop methods that can be used to study these cells *in vivo.* It is clear that the development and use of these methods will be difficult and sometimes less definitive. However, if gliobiologists are to convince the scientific community that astrocytes are major players in brain function, these cells must be studied *in vivo.* In addition to characterizing the properties of these cells *in situ,* it will be necessary to perturb these properties *in vivo* in order to demonstrate their importance in brain function. While certainly more difficult than using culture systems, the sophisticated electrophysiological and molecular biological approaches being applied in neurobiology should lead to major conceptual advances in our understanding of astrocytes *in vivo.*

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24 Peptide receptors on astrocytes

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It is now abundantly clear that astrocytes can express a multiplicity of cell surface receptors rivaling that of neurons. They are able to respond to amino acids, amines, peptides, purines, and prostaglandins. Unlike neurons, however, astrocytes are not connected synaptically, and thus any neuroactive molecules reaching them following release into the synaptic cleft must do so secondarily. Many different types of peptides have been shown to be present presynaptically where they are stored in large, dense-core vesicles. They tend to be present at lower concentrations than the classical neurotransmitters and are probably released at higher frequencies of stimulation (Scheller and Hall, 1992). There is still uncertainty about the mechanism and sites of their release within the presynaptic bouton. Further, whereas the amino acids and amine neurotransmitters are rapidly removed from the synaptic and other extracellular spaces by high-affinity uptake transporters in both neurons and astrocytes, peptides appear to be degraded extracellularly and persist longer than the amino acids or amines. Thus they can both act over longer periods of time and possibly diffuse further than the classical neurotransmitters (Scheller and Hall, 1992). Peptides released from one neuron might well activate receptors on several local glial cells. In addition to neuronally released peptides, other synthetic compartments may also be important. Astrocytes themselves have been shown to be immunoreactive for angiotensin II (Stornetta et al., 1988; Intebi et al., 1990), endothelin (Ehrenreich et al., 1991), preproenkephalin (Vilijn et al., 1988; Shinoda et al., 1989), somatostatin (Shinoda et al., 1989), and substance P (Michel et al., 1986; Kostyk et al., 1989) and thus may release peptides to act in an autocrine or paracrine fashion. Following damage to the central nervous system (CNS) and breakdown of the blood brain barrier (BBB), both blood-borne peptides and those originating in cells of the immune system may also be of importance.

Most of the data that are available so far on glial peptide receptors, have been obtained from experiments using cultured astrocytes. These cells have been usually obtained from neonatal animals and both their developmental and reactive status in culture is unclear. It is important, therefore, to correlate receptor expression in culture with expression *in vivo*. These correlations are beginning to be made, and they will be noted here. Of necessity, in the space available, we have been able to give only a slim overview of a rapidly expanding field of research. We have had to be selective in our choice of representative references and apologize to the authors of many that we have not included.

We first reviewed data about peptide receptors in 1988 (see Wilkin and Cholewinski, 1988). As far as we are aware there has been no further information forthcoming on the effects of calcitonin, corticotropins, glucagon, melanocyte-stimulating hormone, or parathyrin since that time, so readers of this chapter are referred to the previous review for information about those peptides.

ANGIOTENSIN II

The octapeptide angiotensin II (AII) is synthesized both in peripheral tissues and in the CNS. The activation of AII receptors in the circumventricular organs of the brain seems to be responsible for the regulation of blood pressure and water and sodium balance. These receptors respond both to blood-borne and cerebrospinal fluid-derived AII (Ganong, 1983). In addition to the circumventricular receptors, both neurons and astroglia in other brain regions have been found to express AII receptors (Sumners et al., 1990). The physiological function of these receptors is, however, less clear.

The central nervous system possesses an intrinsic renin-angiotensinogen-angiotensin 1-converting enzyme system (RAS) necessary for the synthesis of the peptide (Ganong, 1983). Components of the RAS have been found in both neurons and astrocytes (Kumar et al., 1988; Raizada et al., 1984; Sumners and Raizada, 1984), although recently the latter cell type has become more the focus of attention. Astrocytes appear to possess a full complement of essential components for the synthesis and degradation of AII. Indeed, studies by Intebi et al. (1990) and Stornetta et al. (1988) using both astrocyte cultures and

in situ hybridization showed that astrocytes seem to be the major synthetic compartment for angiotensinogen. Further, Intebi et al. (1990) demonstrated that astrocytes from subcortical regions produced more peptide than cortical ones, a finding consistent with both the *in vivo* distribution of the peptide and the regional heterogeneity of astrocytes (see Wilkin et al., 1990, for references pertaining to heterogeneity). Intebi et al. (1990) go so far as to suggest that astrocytes may even supply angiotensinogen to neurons for further processing.

AII receptors have been divided into two major types: AT_1 and AT_2. This designation was made possible by the availability of AII antagonists such as DuP753 and PD123177, which block AT_1- and AT_2-linked responses, respectively (see Sumners et al., 1991). AT_1 receptor cDNA clones were isolated from both rat (Iwai et al., 1991; Murphy et al. 1991) and bovine (Sasaki et al., 1991) tissues, and subsequently a second form of the AT_1 receptor has been described which was isolated from the rat anterior pituitary (Kakar et al., 1992). AT_{1a} and AT_{1b} receptors display 95% homology. Both astrocytes and neurons possess AII receptors (Sumners et al., 1990). Pharmacological studies have revealed that rat astrocytes possess receptors of the AT_1 subtype (Bottari et al., 1992), while human astrocytes (derived from neoplastic brain) additionally express AT_2 receptors (Jaiswal et al., 1991). Whether the AT_1 receptors are of the subtype a or b awaits analysis, using the newly described molecular probes. In contrast to astrocytes, Sumners et al. (1991) reported that neurons cultured from whole-rat brain expressed predominantly AT_2 receptors, with a lesser number of AT_1s. AT_{1a} and AT_{1b} receptors are differentially expressed in the CNS. Whereas AT_{1a} mRNA is expressed predominantly in the hypothalamus and the median eminence, the cerebellum, subfornical organ, and organum vasculosum of the lamina terminalis have more AT_{1b} mRNA. In contrast, the area postrema had approximately equal quantities of AT_1 subtypes (Kakar et al., 1992). mRNA expression was found to be in good agreement with the presence of AT_1 receptors determined by radiolabeled ligand binding receptor assays and quantitative autoradiography (see Kakar et al., 1992 for references). However, the regional disposition of the particular cell types, astrocytes or neurons, bearing AII receptors has yet to be determined.

Several functional consequences of AII receptor activation have been reported. Sumners et al. (1991) demonstrated that AT_1 receptors on rat astrocytes derived from whole brain were coupled to increased phosphatidylinositol (PI) turnover, whereas neuronal receptors predominantly of the AT_2 type were linked to a reduction of basal cGMP levels. Human astrocytes displayed both AT_1 and AT_2 linked prostaglandin release (Tallant et al., 1991). Whereas the AT_1 receptors were linked to Ca^{2+} mobilization, the AT_2 receptors were not, suggesting that the latter receptors stimulated arachidonic acid mobilization and prostaglandin release through a Ca^{2+} independent mechanism.

A further intriguing function of the AT_1 receptor is its coupling to the release of plasminogen activator inhibitor 1 (PAT-1) from astrocytes derived from 21-day-old rats (Olson et al., 1991; Rydzewski et al., 1992). Astrocytes derived from neonatal animals did not display this response, suggesting that the response may be developmentally regulated. Stimulation with 10 nM AII led maximally to a 20-fold increase in PAI-1 mRNA and release of the protein into the culture medium. The function of the released PAI-1 is at present uncertain, but Rydewski et al. (1992) speculate that it may provide a way of regulating AII action in the brain.

BRADYKININ

Bradykinin (BK) is a nine-amino acid peptide deriving from a precursor, kininogen, following the action of a serine protease, kallikrein. The general interest in this peptide results from the fact that BK is a mediator of pain, inflammation, and hypotension (Dray and Perkins, 1993). It is synthesized in peripheral tissues and in the CNS. The latter appears to have its own endogenous capacity for BK synthesis. Both kininogen and kallikrein have been detected in the brain and BK itself has been localized immunocytochemically and measured quantitatively by radioimmunoassay (Correa et al., 1979; Perry and Snyder, 1984; Kariya et al., 1985). Although these latter quantitative data indicated that the peptide was present in many regions of the rat brain, albeit at relatively low concentrations, recent autoradiographic studies of guinea pig brain showed that binding sites for ^{125}I-BK were restricted to the medulla oblongata (Privitera et al., 1991). Other studies on the regional distribution of ^{3}H-BK binding to membranes of guinea pig brain suggested a wider distribution of receptors and appear to be at odds with the studies of Privitera et al. (Sharif and Whiting, 1991). Further studies should shed more light on these anomalies.

Which CNS cell types are capable of responding to BK? Responses have been reported in brain cortical and thalamic neurons (Phillis and Limacher, 1974; Davis and Dostrovsky, 1988). Cultured rat brain neurons were shown to possess receptors for

^{125}I-BK (Lewis et al., 1985), and cultured rat brain endothelial cells responded to BK with changes in intracellular Ca^{2+} levels (Revest et al., 1991). We found that astrocytes derived from the cortex, cerebellum, and spinal cord (and, more recently, optic nerve and corpus callosum: D. Marriott and G. Wilkin, unpublished observations) of neonatal rat all responded to BK with increased rates of phosphatidylinositol turnover (Cholewinski et al., 1988). Binding studies on the cortical cells with ^{3}H-BK revealed high-affinity binding sites of just one affinity displaying saturability and reversibility (Cholewinski et al., 1991). BK receptors were initially subdivided into types 1 and 2 on the basis of their interactions with a number of antagonists. Using two of these, Des-Arg^{9},[Leu^{8}]-BK (BK-1 antagonist) and D-Arg[Hyp^{3}-,D-Phe^{7}]-BK (BK-2 antagonist) we determined the receptors on astrocytes to be of the BK-2 type (Cholewinski et al., 1991). The rat BK_2 receptor has recently been cloned and sequenced, revealing a predicted structure homologous to the seven transmembrane G protein-coupled superfamily of receptors (McEachern et al., 1991). Now that this information is available, construction of suitable probes for *in situ* hybridization should enable the location of BK receptor-bearing cells to be specified in the CNS.

Binding of BK to astroglial receptors results in several consequences, the induction of a membrane current, the elevation of internal Ca^{2+} and the release of prostaglandins (Gimpl et al., 1992; Wilkin and Marriott, 1993).

ENDOTHELINS

First described in 1988 (Yanagisawa et al.), the endothelin family comprises three 21-amino acid peptides (endothelins 1-3, ET-1-3). All three possess intrachain disulfide bridges, which are also found in the snake sarafotoxins (for review see Greenberg et al., 1992). ET-1 is the most potent vasopressor yet found, causing sustained elevation of blood pressure through its action on smooth muscle cells. The three endothelins, coded for by three separate genes, have been demonstrated in the genomes of human, rat, and pig (Inoue et al., 1989). Although endothelins are present in the human nervous system (Giaid et al., 1989; Lee et al., 1990) the subtype(s) have not yet been defined. Both ET-1 and ET-3 are, however, found in the brains of experimental animals (Matsumoto et al., 1989; Shinmi et al., 1989). In the human CNS, ET mRNA and ET-like immunoreactivity are localized in endothelial cells and neurons (Giaid et al., 1989; Lee et al., 1990). Other reports suggest that astrocytes are also capable of synthesizing endothelins. MacCumber et al. (1990) presented evidence for the expression of mRNA for ET-1 by cultured rat cerebellar astrocytes, although, in contrast, Hama and colleagues (1992) failed to demonstrate its presence. Ehrenreich et al. (1991), however, found that astrocytes from neonatal rat brain displayed ET-3-like immunoreactivity and released the peptide into the culture medium. With respect to endothelial cell synthesis of endothelins, Yoshimoto et al. (1991) found that cerebral microvessel endothelium is capable of producing ET, whereas Vigne et al. (1990) found that they did not. Clearly, much work remains to be done before a full picture of the synthetic compartments for the synthesis of ETs in the CNS is obtained.

cDNA clones for ET receptors have been isolated through the use of direct expression techniques (see Sakurai et al., 1992). Although pharmacological studies have suggested that there may be a number of receptor subtypes (see Gulati and Srimal, 1992, for references), Southern blot analysis of human, pig, and rat genomic DNA has revealed only two distinct genes (Sakamoto et al., 1991). The receptors coded for by these genes have been dubbed ET_A and ET_B. The former is selective for ET-1, whereas the latter displays equipotent affinity for all three ETs and sarafotoxin. An outstanding question is which CNS cell types express which receptor? Autoradiographic studies have shown that binding sites for ETs are found throughout the CNS (Kohzuki et al., 1991). However, the resolution of such studies is such that it is not possible to localize receptors to individual cell types, and therefore, analysis of cultured cells have been undertaken. Hösli and Hösli (1991) demonstrated autoradiographically that both neurons and astrocytes derived from cerebellum and spinal cord expressed binding sites for ^{125}I-ET-1 and 3, and the growing literature suggests that, indeed, both cell types respond to ETs (see references in Gulati and Srimal, 1992).

Hama et al. (1992) demonstrated that ETs act on astrocytes through the ET_B receptor. They found that ET reversed the effects of dibutyryl cAMP by stimulating cell division and suppressing glutamine synthetase activity. ET-induced mitosis has also been demonstrated by Suppatapone et al. (1990) and MacCumber et al. (1990). Several studies have shown that cultured astrocytes respond to ETs with increased phosphatidylinositol turnover and calcium flux (MacCumber et al., 1990; Marsault et al., 1990; Lin et al., 1990; Holzwarth et al., 1992; Morton and Davenport, 1992). One consequence at least of these intracellular messenger changes has been reported to be an inhibition of gap junction permeability

(Giaume et al., 1992). Since their discovery in 1988, there has clearly been a great deal of interest in the ETs; however, much remains to be done, and at this time, it appears that astrocytes may well turn out to be an important functional compartment in the CNS.

NATRIURETIC PEPTIDES

Natriuretic peptides comprise a family of molecules 21-33 amino acids in length. Atrial natriuretic peptide (ANP), the first to be discovered (de Bold et al., 1985), is a peptide of some 28 amino acids. In the brain N-terminally shortened forms exist, a-ANP (4-28) and a-ANP (5-28) (Ueda et al., 1987). Brain also contains two further members of the family, brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) comprising 26 amino acids and 22 amino acids, respectively (Sudoh et al., 1988; Sudoh et al., 1990). In contrast to ANP and BNP, which are concentrated in the heart, CNP is found in high concentrations in the CNS (Ueda et al., 1991). BNP immunoreactivity is more widely distributed throughout the rat brain than is ANP, and is found in all regions of the cerebral cortex and in the amygdala, cerebellum, circumventricular organs and area postrema, hippocampus, and olfactory bulb (Saper et al., 1989). Likewise, CNP is distributed throughout the brain and spinal cord, with particularly high concentrations in the cerebellum, medulla, pons, and hypothalamus (Ueda et al., 1991). Although most earlier studies suggested a neuronal location for natriuretic peptides, recently two reports have appeared demonstrating the presence of ANP in astrocytes. McKenzie (1992) found ANP-like immunoreactivity in astroglia throughout the canine brain. The number of positive cells per unit area of section was usually small, with the exception, however, of a group of cells in one hemisphere. McKenzie suggested that these data might indicate that astroglia express ANP only transiently, perhaps in response to local perturbation, and the small group of ANP-positive cells was the result of such a environmental change. However, a tissue culture study in which all the cells would have been exposed to the same conditions, also showed that only a small number of astrocytes were ANP-immunoreactive (Schipper et al., 1991).

Cloning and sequencing of natriuretic peptide receptors has revealed three separate types, designated GC-A, GC-B, GC-C, and ANPCR. The first three not only act as receptors for peptides, but when activated also synthesize cGMP by virtue of their intracellular catalytic domains. GC-C, however, appears to be a receptor for a bacterial enterotoxin rather than a natriuretic peptide. The fourth receptor does not contain a guanylyl cyclase domain and appears to act as an ANP clearance receptor (for review see Garbers, 1992). Autoradiographic studies of ANP receptors have revealed their presence in various regions of the brain, predominantly, however, in the subfornical organ and other regions without a blood-brain barrier (Kurihara et al., 1987). Within the area postrema of the circumventricular organs, Konrad et al. (1992) demonstrated that ^{125}I-ANP bound to both neuronal processes and astrocytes. In contrast a study by Vente et al. (1990) suggested that astrocytes are the major target for ANP. These workers located ANP-stimulated cGMP immunohistochemically in the olfactory bulb, amygdala, and hippocampus, and in each region the responsive cells were astrocytes. Several studies have also shown that cultured astrocytes respond to natriuretic peptides with increases in cGMP (Friedl et al., 1985; Simmonet et al., 1989; Beaumont and Tan, 1990). A recent study by Yeung et al. (1992) showed that, of the three natriuretic peptides described so far, CNP was the most effective in stimulating cGMP production. As yet, the functional consequences of this increase in cGMP levels within astrocytes are unknown.

OPIOIDS

The interactions of opioid peptides with astrocytes have been studied using cells cultured from cerebral and cerebellar cortices and pituicytes from the posterior pituitary. The actions of opioid peptides and opiate agonists on astrocytes appear to be inhibitory. Both Rougon et al. (1983) and Eriksson et al. (1990) found that opioid receptor agonists antagonized rises in cAMP induced through other receptor types. Pearce et al. (1985) demonstrated that opioid receptors were responsible for the attenuation of an adrenergic-stimulated increase in 2-[^{14}C]deoxyglucose incorporation into glycogen. In a series of studies, Stiene-Martin and Hauser (1990, 1991; Stiene-Martin et al., 1991) demonstrated that opioid receptors are linked to the inhibition of cell division in astrocytes. The receptor types that seem to be important in these phenomena are delta and kappa, rather than those of the mu subclass. All of these receptor types are present in the CNS early in development (Ruis et al., 1991), and the studies of Stiene-Martin and Hauser might suggest that it is during development that opioid actions on astrocytes are of the greatest importance. However, in adulthood, kappa opioid receptors do appear to be

present on a specialized subset of astrocytes, the pituicytes, both in the normal posterior pituitary and following lesion of the neuronal innervation (Bunn et al., 1985). The physiological function of kappa receptor activation is unclear. It has been proposed that pituicytes, through changes in their position relative to oxytocin- and vasopressin-containing terminals, may have a role to play in hormone release (for a recent review see Hatton, 1990). However, while beta-adrenergic ligands induced pituicyte movements both in the isolated gland and in presumptive cultured pituicytes, the kappa ligand dynorphin was without effect (Bicknell et al., 1989; Luckman and Bicknell, 1990). More recent data from this laboratory, however, indicate that dynorphin both delays and reduces vasopressin-induced calcium movements in presumptive cultured pituicytes (R. Bicknell, personal communication). We have used the term *presumptive* with respect to cultured pituicytes in the experiments described above, as their phenotype in culture is debated (Bunn et al., 1990). Burnard et al. (1991) have demonstrated dynorphin-activated electrophysiological changes in pituicytes in isolated rat pituitaries.

OXYTOCIN AND VASOPRESSIN

The two classical neurohormones oxytocin and vasopressin, readily associated in most minds with the hypothalamus, are found in axons that project from this region to the posterior pituitary. Projections to other parts of the CNS have also been mapped (Zimmerman, 1983). In a comparative study of three CNS regions we demonstrated a heterogeneity in the response of astrocytes in respect of phosphatidylinositol turnover (Cholewinski et al., 1988). In each region both peptides gave the same response. The greatest increase was seen in cortical astrocytes (about 150% over basal) followed by cerebellar astrocytes (about 70% over basal) with negligible responses in spinal cord cells. Recent studies by Di Scala-Guenot and Strosser (1992) have characterized the oxytocin receptors on cultured astrocytes from rat hypothalamus and hippocampus. They used an iodinated selective oxytocin antagonist to demonstrate that the receptors were different from those previously reported for neuronal cultures. Few studies have been carried out to examine the possible functions of receptor activation. Del Bigio et al. (1992) found that vasopressin caused a mild swelling of mouse astroglia, and Hatten et al. (1992) demonstrated that vasopressin mobilizes intracellular Ca^{2+} in cultured rat pituicytes, while Hösli et al. (1991) reported membrane depolarization of rat spinal cord and brainstem astrocytes.

SOMATOSTATIN

Somatostatin, comprising 14 amino acids, was initially isolated from the hypothalamus and has been found to act both as an inhibitor of hormone release and as a central neurotransmitter. In the CNS the distribution of the peptide and its receptor is widespread and exhibits regional, quantitative variations (Pelletier et al., 1986; Fitzpatrick-McElligott et al., 1991). Until recently, however, the cellular targets for centrally released somatostatin were completely unclear. A degree of clarification has now been achieved through the use of gold-conjugated somatostatin that retains its binding characteristics. Mentlein et al. (1990) and Krisch et al. (1991) undertook studies of both cultured cells and brain slices to microscopically locate the binding sites for this ligand. In both cases astrocytes featured prominently as a compartment to which somatostatin bound. Not all astrocytes in either the cultures or sections were labeled, but, importantly, in the sections there was a general correlation between the distribution of somatostatin-immunoreactive terminals and the pattern of peptide binding. This suggests that not only are astrocytes regionally heterogeneous with respect to their possession of somatostatin receptors, but they are also located correctly to respond to locally released peptide. A report from Kluxen et al. (1992) suggests that the somatostatin receptor has now been cloned, raising the possibility of *in situ* hybridization studies as another approach to locating receptor-expressing cells.

As stated above, somatostatin generally acts in an inhibitory manner, and its action on astrocytes is no exception. Several studies have shown that it has no effect on basal cAMP levels, but is an effective inhibitor of stimulated increases in this second messenger brought about by a number of different agonists (see Wilkin and Cholewinski, 1988, for references). Beyond these changes in cAMP levels, however, the functional significance of somatostatin effects on astrocytes is unknown.

SUBSTANCE P

Substance P (SP) is an 11-amino acid member of the tachykinin family, which also includes neurokinins A and B (Maggio, 1988). Both the peptide and its

receptors are found throughout the CNS (Helke et al., 1990). Several tachykinin receptors have been cloned and their sequences indicate that they belong to the ever-growing seven transmembrane G protein-linked family (Masu et al., 1987; Yokota et al., 1989; Shigemoto et al., 1990). A great deal of interest has been taken in SP ever since the suggestion of a function in pain pathways, in particular in the dorsal horn of the spinal cord (Jessell, 1983). In comparative studies of the effects of SP on astrocytes derived from neonatal rat cortex, cerebellum, and spinal cord, the latter cells were unique in that they were the only ones to respond to SP with increased phosphatidylinositol turnover (Cholewinski et al., 1988). Subsequently, this heterogeneity was confirmed by Beaujouan et al. (1990) who measured ^{125}I-SP binding sites and found substantial numbers on astrocytes derived from rat brainstem or spinal cord, but low or negligible binding on other CNS-derived cells. In contrast to the data obtained on rat astrocytes, Torrens et al. (1986) had found that murine astrocytes from several CNS regions all possessed high-affinity SP binding sites functionally linked to phosphatidylinositol turnover. These sites have now been shown to be coupled to Ca^{2+} fluxes (Delumeau et al., 1991). Although the reason for this difference between two closely related species is not understood, recent experiments undertaken in our laboratory have revealed the reason for the regional variation in rat brain astrocyte responses to SP. It turns out that the cell types that respond best to SP are the type 2 astrocyte and its precursor the O-2A cell, which derives from white matter tracts (Marriott and Wilkin, 1993). It appears that type 1 astrocytes have very low numbers of receptors, but presumably even low numbers would be enough to invoke the type of membrane changes in K^+ and Cl^- channel opening reported by Backus et al. (1991).

The correlation between SP responsiveness and white matter is intriguing in the light of data from Mantyh et al. (1989), who showed that SP receptors appeared in gliotic lesioned rabbit optic nerve. This appears to be a phenomenon associated with myelinated tracts as Mantyh and Hunt (1986) had previously shown no such upregulation of SP receptors in lesioned gray matter. We now have to determine the cell type responsible for the new expression of SP receptors in damaged optic nerve, but our *in vitro* data suggest that the type 2 astrocyte is a candidate. A consequence of the activation of SP receptors is the release of prostaglandins (Hartung et al., 1988; Marriott et al., 1991) which might be of considerable importance in inflammatory response following white matter damage.

THYROTROPIN RELEASING HORMONE

Although the highest concentration of the tripeptide thyroptropin releasing hormone (TRH) is found in the hypothalamus, quantitatively, more than 70% of total CNS TRH is found outside this region (Reichlin, 1986). The distribution of receptors for this peptide is also widespread throughout the CNS (Manaker et al., 1985). TRH and its dimethylated analogue, RX 77368, have been used in trials to treat patients with motoneuron disease with reported relief of some symptoms (Modares-Sadeghi and Guiloff, 1990). As a part of a study in rats to examine target cells for TRH, we measured its effects on cultured astrocytes derived from neonatal rat cortex, cerebellum, and spinal cord (McDermott et al., 1992). We found relatively modest, but significant, increases in phosphatidylinositol turnover, in all three regions, with the largest increases in the cerebellum. While the regional variations were supportive of astrocyte heterogeneity (Wilkin et al., 1990), the functional significance of the presence of TRH receptors on astrocytes is unknown.

VASOACTIVE INTESTINAL PEPTIDE

Vasoactive intestinal peptide (VIP) is a large peptide of some 28 amino acids first described in 1970 by Said and Mutt. Both VIP and its receptors, mapped using the iodinated molecule, are found throughout the central nervous system (Loren et al., 1979; Martin et al., 1987). The cerebral cortex possesses high levels of both peptide and receptor, and has an interesting population of VIP-positive radially oriented interneurons suggested by Magistretti (1986) to play a role in local glycogenolysis. Although a general picture of the distribution of peptide and receptors is clear, the cell-specific location of receptors is not, prompting a recent study by Martin et al. (1992). They compared the receptor characteristics of astrocytes, microvessels, and synaptosomal membranes from mouse cortex. All three expressed high-affinity binding sites for ^{125}I-VIP, with the neuronal sites differentiated from the nonneuronal ones by the finding that the related peptide, secretin, competed with only the neuronal sites.

Studies in several laboratories have shown that activation of VIP receptors on astrocytes leads to increases in cAMP levels (see Wilkin and Cholewinski, 1988). In a comparative study of rat astrocytes from cortex, cerebellum, and spinal cord we found that by far the greatest response was elicited in the cortical cells, which interestingly parallels the high level of the peptide in this brain region (Cholewinski and Wilkin,

1988; Loren et al., 1979). If the *in vitro* data reflect the *in vivo* situation, then we might predict that cortical astrocytes in the brain will be shown to express sufficient numbers of VIP receptors to enable them to respond effectively to local neurons. Downstream of cAMP synthesis VIP promotes glycogenolysis both in cultured astrocytes (Sorg and Magistretti, 1991) and in cerebral cortical slices (Schaad et al., 1989) suggesting a function for the peptide in the local regulation of energy metabolism. Other astrocytic functions have also been determined by Brenneman and colleagues (1990) who demonstrated that VIP is a secretagogue for a neuron survival-promoting activity and also a mitogen for cultured astrocytes.

CONCLUSIONS

Clearly, astrocytes now have to be seriously considered as potential targets for neuropeptides. It is necessary for the time being to continue to use the word potential, because most of the data derive from studies on cultured cells. Nonetheless, the results of Krisch et al. (1991) and Mentlein et al. (1990) on somatostatin receptor labeling and the dynorphin-induced physiological responses of pituicytes of Burnard et al. (1991) point the way forward. It is now important to investigate the localization of astrocyte peptide receptors within the substance of the CNS proper. We look forward to understanding the relationships between astrocyte receptor expression and local neuronal populations both during development and in the adult, and the changes that may result from reactive gliosis. Undoubtedly, further receptor cloning and the raising of novel antibodies to the various receptors will have a considerable bearing on our progress.

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25 Eicosanoid, purine, and hormone receptors

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Research into glial cell receptors has focused mainly on characterizing receptors for the major classes of signal molecules released from neurons: amines, amino acids and peptides. However, these cells also appear to express receptors for a variety of other substances that may not be solely derived from neurons. Among these are the purines and eicosanoids, agents that might be released from sources such as other glia, neurons, or cells of the vasculature. Although we are far from fully understanding the roles played by these agents in the central nervous system (CNS), involvement in inflammatory and immune responses and regulation of the vasculature are prime candidates. Glia are also targets for steroid hormones, thyroid hormones, insulin, and insulin-like growth factors. Some of these may be synthesized locally, while others may enter the CNS from the circulation. As a general rule, the site of action of these hormones is the nucleus, the responses they regulate being primarily long-term events, such as cell growth, differentiation, and metabolism.

The aim of this chapter is to summarize the evidence for the presence of receptors for this diverse group of signal molecules on glia. This will be derived predominantly from studies of cultured cells; however, attention will be drawn to studies on *in situ* glia. We will also consider the transduction mechanisms coupled to these receptors and the downstream consequences of receptor activation.

EICOSANOID RECEPTORS

All eicosanoids are synthesized from a common precursor, arachidonic acid, via a sequence of enzymatic reactions along either the cyclooxygenase or lipoxygenase pathways to yield prostaglandins (PGs) and leukotrienes, respectively. It has been known for some time that eicosanoids are synthesized within the CNS and are likely to be involved in mediating a variety of cellular events (Wolfe, 1982; Chiu and Richardson, 1985). Studies on cultured cells have suggested that glia and astrocytes in particular may be a major source of eicosanoids in the brain (Murphy et al., 1988). Glia are also targets for these agents, indicating that eicosanoids may be involved in both paracrine and autocrine functions.

Early indications for the presence of eicosanoid receptors on glia came from studies on whole brain cultures and clonal cell lines. In their review of 1980, van Calker and Hamprecht outlined experiments showing that PGE_1, -E_2, and -I (prostacyclin) were capable of activating adenylate cyclase and increasing intracellular cyclic adenosine monophosphate (AMP) concentrations, whereas PGA_1, -A_2, -B_1, -$F_{1\alpha}$, and -$F_{2\alpha}$ were all without effect. With the advent of techniques to produce cultures enriched in either astrocytes or oligodendrocytes, McCarthy and de Vellis (1980) reported that both cell types responded to PGE_1 with an increase in cyclic AMP but that this effect was much greater in oligodendrocyte-enriched cultures. More recent studies have shown that some PGs act on receptors coupled to phosphoinositide breakdown and intracellular Ca^{2+} mobilization.

Both thromboxane A_2 (TXA_2) and $PGF_{2\alpha}$ have been found to promote inositol phospholipid metabolism in cultured cells. Nakahata et al. (1989, 1992) demonstrated the presence of a single, high-affinity binding site for TXA_2 on both human 1321N1 astrocytoma and primary cultures of adult rabbit cerebral cortex. Stimulation of these receptors with a stable TXA_2 analogue resulted in the accumulation of intracellular inositol phosphates in both cell types (Nakahata et al., 1989, 1992). Similarly, Kitanaka et al. (1991) found that $PGF_{2\alpha}$ stimulated phosphoinositide breakdown in rat cortical astrocytes. The receptors for TXA_2 and $PGF_{2\alpha}$ appear to be coupled to phospholipase C, the enzyme responsible for catalyzing phosphoinositide metabolism, via a regulatory G protein which is not sensitive to pertussis toxin (Kitanaka et al., 1991; Nakahata et al., 1989, 1992).

Interestingly, $PGF_{2\alpha}$-evoked phosphoinositide breakdown can be modified by prior treatment of cultures with dibutyryl cyclic AMP. This is a well-established method of transforming protoplasmic astrocytes into cells with a stellate morphology. Under

these conditions $PGF_{2\alpha}$-stimulated inositol phosphate accumulation was enhanced in cortical, hippocampal, and particularly cerebellar astrocyte cultures (Kitanaka and Baba, 1992). Whether this reflects an increase in PG receptor number or is a function of cell differentiation is unclear. However, differences in PG-evoked responses have recently been noted in cultures enriched in protoplasmic and stellate astrocytes prepared from newborn rat cerebral cortex. In both cell populations PGE_1 and $-E_2$ were found to increase intracellular cyclic AMP levels, but the responses were much greater in cells with a stellate morphology (Ito et al., 1992). Apparently, these differential effects were not due to varying adenylate cyclase activities because forskolin, a direct activator of this enzyme, caused similar responses in both cell types. Ito et al. (1992) also examined changes in intracellular Ca^{2+} concentration in cells loaded with the fluorescent indicator fura-2AM (see Chapter 17, this volume). When challenged with $PGF_{2\alpha}$, $-D_2$ and a TXA_2 analogue, only protoplasmic astrocytes responded with an increase in internal Ca^{2+}, an effect that was insensitive to pertussis toxin. Even though stellate astrocytes were unresponsive to these eicosanoids, internal Ca^{2+} could be mobilized by other agents, such as histamine (Ito et al., 1992).

As previously mentioned, eicosanoids are synthesized from arachidonic acid, a constituent of many membrane phospholipids. There is increasing evidence that arachidonic acid itself may serve as a signal molecule; indeed, Murphy and Welk (1989) have shown that it stimulates phosphoinositide breakdown in cortical astrocytes (see Chapter 34, this volume). This effect was found not to be secondary to arachidonic acid metabolism, nor was it the result of its insertion into the membrane; the suggestion was that its action may be receptor-mediated.

Despite the evidence for the presence of eicosanoid receptors on glia in culture, there are few indications of either their functions or whether they exist on cells *in situ*. McCarthy et al. (1985) have shown that at least one of the downstream consequences of PGE_1 receptor activation in astrocytes is the phosphorylation, presumably via a cyclic AMP-dependent protein kinase, of the intermediate filament proteins glial fibrillary acidic protein (GFAP) and vimentin. One might therefore suggest that certain eicosanoid receptors may be involved in cytoskeletal organization during astrocyte differentiation. In addition, Sawada et al. (1993) reported that endogenously synthesized prostanoids regulate astrocyte proliferation. Whether these receptors serve a similar function in oligodendrocytes remains to be determined. Another area still to be fully explored is whether glia possess receptors for leukotrienes. We know that astrocytes are capable of synthesizing these agents (Hartung and Toyka, 1987), but, to our knowledge, there is no evidence for the existence of their receptors on these cells.

PURINE RECEPTORS

Extensive pharmacological characterization of purinergic receptors has led to them being classified as either P_1 or P_2 purinoceptors. P_1 receptors are activated by adenosine and can be further subdivided into A_1 and A_2 receptors on the basis of various functional criteria. A_2 receptors are positively coupled to adenylate cyclase, whereas A_1 receptors have been shown to be either negatively linked to adenylate cyclase or coupled to phosphoinositide breakdown. P_2 purinoceptors recognize adenosine triphosphate (ATP) and can also be divided into various subtypes. The P_{2X}, P_{2Y}, P_{2T}, and P_{2Z} purinoceptors can be distinguished according to agonist selectivity, the cell types possessing receptors and the response initiated upon stimulation.

P_1 *Purinoceptors*

Several lines of evidence point to there being P_1 purinoceptors of both A_1 and A_2 subtypes on glia. Receptors have been identified autoradiographically in explant cultures of rat cerebellum and spinal cord (Hösli and Hösli, 1988) and agonist-induced decreases (A_1) and increases (A_2) in cyclic AMP formation have been observed in a number of primary culture preparations (van Calker and Hamprecht, 1980; Barnes and Thampy, 1982; Ebersolt et al., 1983). The work of McCarthy and de Vellis (1980), however, suggests that the A_2 receptor subtype may be located predominantly on astrocytes.

Recently, attention has focused on glial A_1 receptors. In striatal and mesencephalic, but not cortical, astrocytes, 2-chloroadenosine, a stable adenosine analogue, was found to potentiate α_1-adrenoceptor-induced phosphoinositide metabolism without having activity itself (El-Etr et al., 1989). Delumeau et al. (1991) found that a combined addition of A_1 and α_1-adrenergic agonists to striatal astrocytes produced a sustained increase in intracellular Ca^{2+} concentration, this response being far greater than that caused when the agonists were used separately. Part of this change in internal Ca^{2+} appeared to be due to the receptor-stimulated influx of extracellular Ca^{2+} (Delumeau et al., 1991). These authors have suggested that A_1 receptors are coupled, via a pertussis toxin-sensitive G protein, to phospholipase A_2 and that their actions on phosphoinositide metabolism and Ca^{2+} flux are indirect, being mediated by

A_1 receptor-evoked arachidonic acid liberation (El-Etr et al., 1992).

Researchers have examined some of the functional correlates of P_1 purinoceptor activation in cultured glia. In common with other agents that elevate intracellular cyclic AMP in these cells, adenosine has been shown to promote GFAP and vimentin phosphorylation (McCarthy et al., 1985) and glycogen breakdown (Magistretti et al., 1983) in astrocytes presumably via the activation of the A_2 receptor subtype. Hösli et al. (1987) reported that glia in explant cultures were hyperpolarized by adenosine. The receptor subtype involved in this response is not known, but an ability to modulate membrane K^+ ion conductance seems likely.

P_2 Purinoceptors

The first indication that extracellular ATP had an effect on glia came from the work of Trams (1974). In clonal cell lines ATP was found to alter membrane permeability to various ions, an effect attributed to its action on membrane-bound ectoenzymes. It is now clear, however, that glia possess specific receptors for ATP. In cultured cortical astrocytes ATP induces the breakdown of phosphoinositides via its interaction with P_2 purinoceptors (Pearce et al., 1989). Subsequent studies have concentrated on receptor-mediated intracellular Ca^{2+} mobilization in these cells. Using fura-2AM-loaded astrocytes, it has been shown that approximately 50 to 60% of both protoplasmic and stellate cortical astrocytes respond to ATP with an increase in cytosolic Ca^{2+} concentration, an effect mediated by the P_{2Y} class of receptors (Dave et al., 1991; McCarthy and Salm, 1991; Kastritsis et al., 1992). This response was characterized by a rapid, transient release of stored Ca^{2+}, followed by a more sustained influx of extracellular Ca^{2+} (Kastritsis et al., 1992). The dependence of this part of the response on extracellular Ca^{2+} tends to confirm the earlier work of Neary et al. (1988), who found that ATP promoted radiolabeled Ca^{2+} uptake into these cells. Although the evidence for P_{2Y} purinoceptors coupled to phosphoinositide metabolism is quite strong, electrophysiological studies suggest that glia may possess a subset of P_2 receptors that are linked to membrane ion channels. ATP has been shown to depolarize cultured microglia (Kettenmann et al., 1993) and astrocytes (Magoski and Walz, 1992). This phenomenon has been best characterized in astrocytes where ion substitution experiments revealed that this response was mediated by changes in Na^+ and K^+ flux and the use of various agonists showed a pharmacological profile that does not correspond to any of the accepted P_2 purinoceptor subtypes (Magoski and Walz, 1992).

Functional correlates of P_2 purinoceptor activation have been confined to studies on astrocytes where they appear to be involved in regulating protein phosphorylation and eicosanoid release. Neary et al. (1991) reported that the phosphorylation patterns of four specific proteins were altered in astrocytes challenged with ATP. Two of these proteins, one of which was identified as GFAP, showed enhanced phosphorylation, while in two others phosphorylation was reduced. Thus, P_2 purinoceptors appear to be involved in modulating the activity of Ca^{2+}-dependent protein kinases and phosphatases. With regard to eicosanoid release, ATP has been found to stimulate the release of PGD_2 (Gebicke-Haerter et al., 1988) and TXA_2 (Pearce et al., 1989) from rat cortical astrocytes and TXA_2 from human glioma (Murphy et al., 1990). Bruner and Murphy (1990) have shown that arachidonic acid liberation, and by implication eicosanoid release, is mediated by P_{2Y} purinoceptors, which corresponds to the findings on phosphoinositide metabolism and Ca^{2+} mobilization discussed earlier. However, there is evidence to suggest that eicosanoid release may not be entirely dependent upon ATP-stimulated phosphoinositide breakdown but may involve either the direct coupling of receptors to phospholipase A_2 and/or the activation of a membrane Ca^{2+} channel (Seregi et al., 1992; Bruner and Murphy, 1993).

THYROID HORMONE RECEPTORS

The important role played by thyroid hormones during brain development is well established (Grave, 1977). It is perhaps not surprising, therefore, that these hormones are involved in regulating various aspects of glial maturation. Indeed, it has been shown that thyroid function can markedly influence the development of glial cells in the intact brain (Clos et al., 1980; Pesetsky, 1983).

The action of thyroid hormones is believed to be due to the binding of triiodothyronine (T_3) to receptors in the nucleus of target cells and ligand binding studies have established the presence of T_3 receptors in nuclear fractions prepared from cultured glia (Luo et al., 1986; Puymirat and Faivre-Bauman, 1986). Using a monoclonal antibody raised against rat liver T_3 receptors, Luo et al. (1989) have shown immunocytochemically the localization of T_3 receptors in the nuclei of both protoplasmic and stellate astrocytes prepared from newborn rat cerebral cortex. Most of the cells in these cultures were labeled; however, the receptors appeared more slowly over time

in culture in protoplasmic astrocytes compared to those with a stellate morphology.

The consequences of T_3 receptor activation strongly support a role for these receptors in regulating glial differentiation. Various aspects of myelin production are stimulated by T_3 in oligodendrocyte cultures (Shanker et al., 1985; Koper et al., 1986). In addition, the activity of the astrocyte marker enzyme glutamine synthetase (GS) is influenced by treatment with thyroid hormone, as is the transformation of astrocytes from a protoplasmic to a stellate form (Aizenman and de Vellis, 1987).

STEROID HORMONE RECEPTORS

The brain contains receptors for both adrenocorticoids and those hormones (progestins, estrogens, androgens) usually associated with reproductive functions (McEwan et al., 1982). These receptors are molecularly similar to those found in non-neural tissues and appear to be involved in the regulation of CNS development and behavior.

Androgen, Progesterone, and Estrogen Receptors

Cultured glia, particularly oligodendrocytes, are known to be capable of synthesizing steroids such as pregnenelone and progesterone (Jung-Testas et al., 1989). These authors have also presented evidence for the presence of receptors for these hormones on cultured cells. In cultures containing both oligodendrocytes and astrocytes, Jung-Testas et al. (1991) demonstrated specific binding of ligands to estrogen (ER), androgen (AR), and progesterone (PR) receptors. Estimates of the number of receptor sites per cell indicated that the ER were the predominant species, being almost twofold greater in number than either the AR or PR. Further studies revealed that the number of PR, but not AR or ER, in these cultures could be markedly increased by prior treatment of the cells with estradiol (E_2). Jung-Testas et al. (1991) also showed that staining with an antibody against the PR was confined to the inner nuclear membrane and occurred primarily in oligodendrocytes. These findings were essentially confirmed in a later paper by this group in which cultures enriched in either astrocytes or oligodendrocytes were used. Astrocytes were found to possess very few PRs; moreover, those that were present appeared to be confined to cells derived from female animals and could not be increased in number by E_2 treatment (Jung-Testas et al., 1992). In contrast, oligodendrocytes prepared from both male and female animals possessed PRs and, although more abundant in cultures from females, receptors in cells from both sexes were increased by exposure to E_2 (Jung-Testas et al., 1992).

Functional studies suggest that these steroid hormone receptors play a role in glial cell development. Garcia-Segura et al. (1989) showed that E_2 induced hippocampal astrocytes to adopt a stellate morphology. In addition, treatment of mixed glial cultures with either E_2 or progesterone resulted in changes in the expression of GFAP and myelin basic protein (MBP), these responses being particularly evident in immature cultures (Jung-Testas et al., 1992). Interestingly, Torres-Aleman et al. (1992) have recently shown that the E_2-induced differentiation of hippocampal astrocytes can only occur when the cells are in contact with neurons.

In recent years, our view of steroid hormone receptors being in the cytoplasm and/or nucleus of cells, and the effect of the hormone being mediated via mRNA synthesis and protein expression, has changed. Evidence now suggests that steroid hormones can also elicit rapid nongenomic effects via interactions with membrane-bound receptors. Various metabolites of progesterone and deoxycorticosterone are capable of influencing the activity of both inhibitory and excitatory amino acid receptors in the CNS (Paul and Purdy, 1992). Probably the best studied are those steroids that bind to a site associated with the $GABA_A$-Cl^- ion channel receptor complex. It has been known for some time that cultured glia possess $GABA_A$ receptors (Kettenmann et al., 1988; see Chapter 21, this volume). Recently, Chvatal and Kettenmann (1991) have shown that GABA-mediated membrane currents in cultured cortical astrocytes can be both positively and negatively modified by steroids in a manner similar to their actions on neurons. The functional implications of these effects, however, remain to be elucidated.

The question of whether glia *in situ* possess steroid receptors is open to debate. Autoradiographic and *in situ* hybridization studies indicate that AR and ER do not exist on glia in adult rat brain (Keefe et al., 1991). However, glia in the intact CNS do seem able to respond to both the administration of these hormones and the manipulation of their levels in living animals. For example, the administration of E_2 to adult female rats and the alteration of neonatal levels of estrogens and androgens in male and female rats caused changes in astrocyte morphology and GFAP expression in various brain regions (Garcia-Segura et al., 1986, 1988). These authors have suggested that glial responses to sex hormones may be important in determining synaptic remodeling and the sexual differentiation of the brain.

Adrenocorticoid Receptors

Adrenocorticosteroids activate two classes of intracellular receptor, the mineralocorticoid (MR) or type I receptor and the glucocorticoid (GR) or type II receptor. These receptor classes can be distinguished on the basis that the MR displays a higher affinity for corticosterone than does the GR, which preferentially binds synthetic glucocorticoids such as dexamethasone (Ruel and de Kloet, 1985). There is abundant evidence that cultured glia possess both types of receptor and that they are involved in mediating a variety of cellular events.

Ligand binding studies on cultured glia point to the existence of a single population of GRs in both astrocytes and oligodendrocytes (Chou et al., 1991; Kumar and de Vellis, 1988). These receptors appear to be located in the cytoplasm, but, as immunocytochemical studies have shown, they migrate to the nucleus in the presence of a receptor ligand (Bohn et al., 1991). Chou et al. (1991) have also reported that MRs are present in astrocytes. Similar binding studies have not been carried out on purified oligodendrocyte cultures; however, Bohn et al. (1991) have confirmed the presence of MRs in these cells using antibody labeling.

Nothing is known of the role played by MR in glia; however, the functional correlates of GR stimulation are well documented. Glucocorticoids are known to modulate the expression of a variety of glial proteins, for example, the expression of GS and MBP, and glycerol phosphate dehydrogenase is enhanced in cultured cells (Kumar and de Vellis, 1988). Warringa et al. (1987) have suggested that GR activation may be involved in determining whether bipotential glial precursor cells develop into either astrocytes or oligodendrocytes.

Evidence for the presence of these receptors in glia *in situ* is mainly indirect, although low levels of GR mRNA have been detected in white matter cells (Aronsson et al., 1988). It is clear, however, that glia in the intact brain can respond to glucocorticoids. Adrenalectomy results in increased myelination (Meyer and Fairman, 1985), while glucocorticoid administration inhibits myelination (Gumbinas et al., 1973), the genesis of oligodendrocytes (Bohn and Friedrich, 1982), and the expression of GFAP (O'Callaghan et al., 1989).

INSULIN AND INSULIN-LIKE GROWTH FACTOR RECEPTORS

Insulin and the insulin-like growth factors (IGF) I and II are structurally related molecules. The IGFs are synthesized within the brain, but it is not entirely clear whether the same is true for insulin (Masters et al., 1989); nonetheless, receptors for all of these substances have been found in various central nervous system regions (Gammeltoft et al., 1984; Masters et al., 1989). We are still some way from fully understanding the roles these agents play in the central nervous system, although it is generally accepted that they are likely to be involved in regulating cellular development and metabolism (see Chapter 32, this volume).

Probably the first demonstration of glial insulin receptors came from binding studies on astrocyte-enriched fractions prepared from adult rat brain (Albrecht et al., 1982). Subsequently, a number of workers have reported similar findings from experiments performed on cultured astrocytes (Kum et al., 1987; Clarke et al., 1988). Astrocyte insulin receptors are similar to their neuronal and liver counterparts in that they possess an α and β subunit; however, these subunits have molecular weights intermediate between the neuronal and peripheral receptor types probably because of differences in attached carbohydrate moeties (Clarke et al., 1988). Despite these differences, astrocyte insulin receptors appear to have similar requirements for the structure of the ligands they bind, particularly in relation to the C terminus of the B chain of the insulin molecule (Zhu et al., 1990). Having outlined these studies, it should be noted that Han et al. (1987) found only low levels of specific insulin binding to cortical astrocytes. They did, however, find binding sites for IGF-I and IGF-II on these cells; moreover, autoradiography revealed that these receptors are membrane-bound, but are subsequently internalized and migrate toward the nucleus (Han et al., 1987). Masters et al. (1991a) and McMorris et al. (1986) have shown that IGF receptors are also present on oligodendrocytes and bipotential precursor cells.

There is a considerable body of evidence pointing to insulin and the IGFs being glial mitogens. Insulin, IGF-I, and IGF-II have all been reported to promote precursor incorporation into DNA or to increase cell numbers in glial cultures (McMorris et al., 1986; Ballotti et al., 1987; Han et al., 1987; Kum et al., 1987; Shemer et al., 1987; Clarke et al., 1988; Masters et al., 1991a). In those studies where ligand potencies have been established, it would appear that IGF-I receptors are probably responsible for controlling glial proliferation. Insulin may also be involved in regulating glial differentiation, although reports in the literature are sometimes contradictory. For example, it has been shown that insulin is capable of influencing the phenotypic appearance of astrocytes and the expression of mRNA for GFAP

and its encoded protein in cerebellar organotypic cultures (Toran-Allerand et al., 1991). In contrast, Aizenman and de Vellis (1987) found that insulin had no effect on astrocyte morphology in primary cultures. These authors also reported that insulin and hydrocortisone were synergistic in their ability to induce GS expression in rat cortical astrocytes (Aizenman and de Vellis, 1987); however, Tholey et al. (1986) demonstrated the exact opposite in embryonic chick brain astrocytes. Whether this discrepancy is due to the different species of animal used or its developmental stage is not clear.

In peripheral tissues, insulin is well known to be involved in regulating cell metabolism. Studies on cultured cells suggest that glial glucose utilization may be similarly controlled by insulin and IGFs. Insulin at low nanomolar concentrations stimulates the uptake of glucose into astrocytes (Clarke et al., 1988) and its subsequent incorporation into glycogen (Dringen and Hamprecht, 1992; Kum et al., 1992). Shemer et al. (1987) suggested that glucose uptake may be a function of insulin rather than IGF-I receptor activation. However, Dringen and Hamprecht (1992) have shown that IGF-I is more potent than insulin in inducing glycogen formation in astrocytes. This metabolic response may, however, be developmentally regulated because Masters et al. (1991b) reported that IGF-I only activates the glucose transporter in cultures prepared from newborn animals; in cultures of adult tissue, IGF-I was found to be ineffective.

The intracellular transduction mechanisms linked to insulin and IGF receptors have yet to be fully characterized. There is evidence that these receptors may be coupled to a tyrosine kinase; indeed, insulin- and IGF-evoked kinase activation has been demonstrated in cultured glia (Clarke et al., 1988; Masters et al., 1989). However, this is unlikely to be the only signaling pathway operated by these molecules. Roles for protein kinase C and an as yet unidentified cytosolic factor that is involved in the activation of a regulatory ribosomal protein have also been suggested (Masters et al., 1989).

CONCLUSIONS

The list of receptors known to be present on cultured glial cells is growing steadily. We are beginning to characterize the signal transduction pathways coupled to these receptors and the biological consequences of receptor activation. As always, however, we are faced with the question: do these receptors exist on glia in the intact brain? In some cases it is probable that they are expressed in *in situ* glia, particularly receptors for certain hormones, insulin and/or IGF, and so on, where their presence may be developmentally regulated and involved in glial maturation and differentiation. Whether this is also true for purine and eicosanoid receptors is still open to debate. Although the evidence points to these agents serving as signal molecules in the CNS, it remains to be established whether glia in the normal brain are targets for them. It is conceivable that the expression of these receptors in cultured cells is telling us something about the status and possible functions of glia in the damaged brain. The removal of glia from their connections with neurons, other glia, the vasculature, and so on, and into the culture dish, might be an approximation of the situation they face during inflammatory conditions or vascular insults. This raises the interesting and as yet unanswered question of whether glia express certain types of receptors in response to changes in their physical and/or chemical environment.

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V Mechanisms of Cell-to-Cell Communication

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26 Extracellular space as the pathway for neuron-glial cell interaction

CHARLES NICHOLSON

This chapter will deal with current concepts of the extracellular space, how it can be defined in terms of a small number of biophysical parameters, and how these parameters enable us to explore the migration of substances between neurons and glia. The question of length scale will be discussed using a simple general model based on recent analyses of diffusion in the brain and a model applicable to very short-range interaction based on axon-Schwann cell interaction. An overview of the spatial-buffering hypothesis for potassium will be presented as an example of a mechanism where glial cells are thought to play a major role.

EXTRACELLULAR SPACE AND THE PARAMETERS THAT DEFINE IT

It is now appreciated that the space between cells of the brain is not simply an empty region separating membranes, but a complex environment that likely plays an important role in many processes that occur in the brain. The term *brain extracellular microenvironment* (BEM) will be used here, designating the environment encountered by a molecule when that molecule is in the space between cells of the brain. A similar term *brain cell microenvironment* was coined by Schmitt and Samson in a workshop as far back as 1969. In the intervening years it has become necessary to distinguish the extracellular from the intracellular microenvironment so that the term BEM is now more appropriate. Further information on this topic can be found in Nicholson (1980), Schmitt (1984), and Cserr (1986).

The idea of nonsynaptic communication between cells (Nicholson 1979) has been discussed most recently under the name of volume transmission (Fuxe and Agnati, 1991) and nonsynaptic diffusion neurotransmission (Bach-y-Rita, 1993). Numerous papers have provided evidence for the transmission of chemical signals between glia and neurons (a few randomly chosen recent examples: Stone and Ariano, 1989; Butt, 1991; Evans et al., 1991; Grimaldi et al., 1991; Grossfield et al., 1991; Lierberman, 1991; Martin, 1992; Lieberman et al., 1993). In Chapter 31 in this book, Orkand discusses aspects of this issue at greater length. The purpose of this chapter is to consider what constraints the biophysical properties of the extracellular microenvironment impose on the movement of substances between cells.

Volume Fraction, Tortuosity, and Uptake

A useful way to think of the extracellular space, in the words of Kuffler and Potter (1964), is that "... these spaces ... in three dimensions ... resemble the water phase of a foam." This is illustrated in Figure 26-1A. A substance released from a neuron or glial cell enters a restricted space where it is hindered in its diffusion by the presence of numerous cell membranes; finally, the substance may be actively taken up and modified or metabolized at some of the membrane surfaces.

It is now clear that the fraction of brain volume that is extracellular space amounts to 20% of the total brain volume. This rather large value arises because every area of cellular membrane is associated with an "atmosphere" of extracellular space so that the immense amount of cellular membrane per unit volume in brain tissue leads naturally to an appreciable volume.

While volume fraction is an important determinant of the distribution of substances in the BEM, it is not the only one. A somewhat less intuitive, but equally important parameter is tortuosity. This parameter measures the extent to which the cellular obstructions of the brain hinder the movement of substances. For a long time studies concentrated on either volume fraction or tortuosity but did not determine both, or in some cases even failed to recognize them as separate entities (for further discussion see Nicholson and Phillips, 1981).

Both volume fraction and tortuosity are morphological parameters and, in ideal measurements, are

independent of the substance or method used to measure them. A third, substance-specific, aspect of the BEM is the uptake property or the extent to which a given substance is removed from the extracellular space. Because of the high membrane:extracellular volume ratio in the BEM a certain amount of nonspecific loss of any substance occurs, and this generally is linearly related to extracellular concentration. In many cases, however, material is actively transported across membranes by an energy-dependent uptake system that obeys nonlinear kinetics or the substance may be metabolized *in situ*.

These three factors—volume fraction, tortuosity and uptake—determine how far substances will move in the extracellular space and so have the potential to play a major role in glia-neuronal interaction.

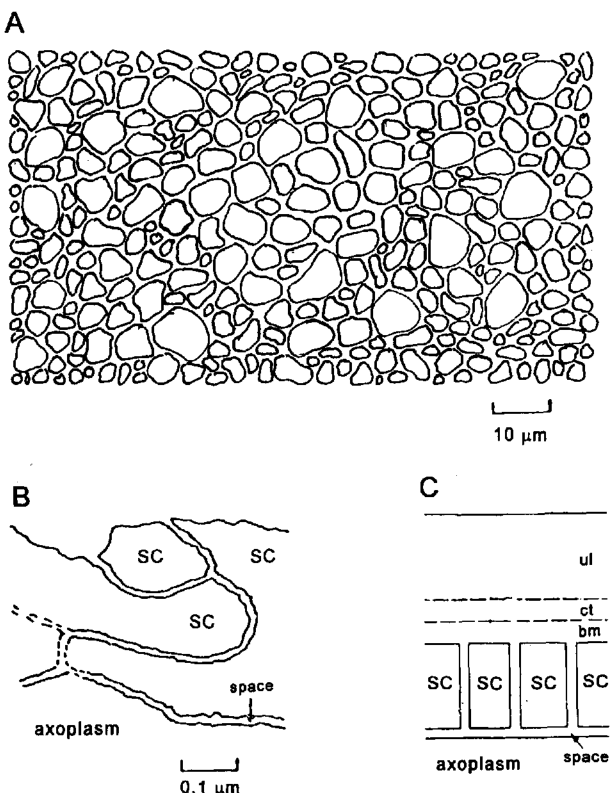

FIG. 26-1. Geometry of the extracellular space at different length scales. (A) Geometry appropriate for a scale of greater than 1 μm. The closed profiles represent cell bodies, dendrites, glial processes, and axons. The space between cells resembles the liquid phase of a foam. Substances are released into a restricted volume, defined by the volume fraction α and hindered in their movement through the brain by cellular obstructions, which constitute a tortuosity factor, λ. In addition, uptake or degradation of molecules occurs at membrane surfaces. (B) Relationship between a giant axon and surrounding Schwann cells. Models based on this geometry seek to account for behavior of substances in the space between the axon and immediate Schwann cells (*SC*) and are applicable on a length scale of less than 1 μm. In this model the primary route for escape of substances from the space was envisaged to be the clefts between cells. (C) A later representation of the geometry shown in Figure B. The Schwann cells are now represented by rectangular cells separated by regular clefs. Beyond these lie a basement membrane (*bm*), connective tissue (*ct*) and an unstirred layer (*ul*). See text for further details. [Figure B modified from Frankenhaeuser and Hodgkin (1956), with permission; Figure C modified from Taylor et al. (1980), with permission.]

Spatial Scale and Diffusion

In discussing neuron-glia interaction it is important to consider the spatial scale involved. When distances of tens of micrometers are considered, the morphological parameters are subsumed into the three factors described in the preceding section (Figure 26-1A). On the other hand, some questions revolve around the interaction of a neuron with its surrounding glial sheath. In axon-Schwann cell interactions, for example (Figures 26-1B,C), local morphology will play the dominant role. The diffusion of substances over varying distance scales is differently affected by the geometry of the BEM and by uptake processes.

As an example of this interaction, to be described below, glial cells have a special mechanism for removing the K^+ released by neurons from the BEM. Over short distances the movement of K^+ is mainly limited by diffusion but over long distance a novel form of uptake known as spatial buffering comes into play and both these processes are subject to further modulation by active transport of the K^+ by either neurons or glia.

Dynamic Aspects

The facets of the BEM briefly mentioned above show great potential for dynamic behavior. The net movement of solutes across membranes leads inexorably to water movement and with it changes in cell volume and alterations in the architecture of the extracellular space (e.g., Nicholson, 1980; Dietzel et al., 1989). Such changes may be of functional importance or they may play a major role in pathophysiology. In a real sense the whole structure of the BEM, and indeed the brain itself, is a dynamic entity that is sustained by the continuous consumption of energy. This behavior may be modulated by neuroactive factors. A discussion of the role of glia in glucose metabolism is presented in Chapter 52. Our emerging picture of the BEM is that of a dynamic entity whose properties can change with time, and the presumption is that glia play a role in this behavior.

VOLUME FRACTION

The volume fraction of the BEM is defined as α, the ratio of the extracellular phase of brain tissue to the total tissue phase (which comprises extracellular phase, intracellular phase, and boundary or membrane phase) of a small volume of brain tissue. The volume fraction is therefore a dimensionless ratio.

It was not always believed that the percentage volume fraction of the BEM was 20%, indeed early estimates, based on electron microscopy suggested that it was no more than 5% (Horstmann and Meves, 1959). Other methods of estimating the percentage volume at that time, such as impedance and radiotracer measurements with Na^+ or Cl^-, gave very variable results so that the true percentage was a matter of debate for a number of years. One of the earliest investigators to accurately estimate the percentage volume was van Harreveld (1972), who used a variety of techniques, including electron microscopic methods, that attempted to preserve the spaces between cells.

Consistent estimates of extracellular volume fraction, as it has come to be known, became available with the use of careful quantitative radiotracer methods based on molecules that remained extracellular. Thus Levin et al. (1970), using labeled sucrose and inulin, obtained values of 18 to 20% in the cerebral cortex of four mammalian species. Most recently, the local iontophoresis of TMA^+ and an analysis of its diffusion characteristics have provided accurate measurements in the living brain (Table 26-1). This method provides sufficient temporal resolution that changes in volume fraction can be followed in real time.

One possible reason why the volume fraction cannot be negligible is because it has to act as a conduit, between blood vessel or ventricular surface and cell surface, for metabolic substrates and waste products. When the brain is subjected to anoxic insult, the extracellular volume fraction decreases (van Harreveld, 1972; Lundbæk and Hansen, 1992; Syková et al., 1994), due presumably to the swelling of cells but never totally occludes, even during ischemic conditions where it reaches a minimum volume of about 5%.

It is also striking (see Table 26-1) that the volume fraction is preserved across species. Thus similar values are reported in fish, reptiles, and mammals. Even more impressive is that similar values can be seen in cephalopod invertebrates. Cephalopods are represented by the octopus, squid, and cuttlefish and have sufficient brain mass to permit measurements of α. Both radiotracer studies (Abbott et al., 1985) and TMA^+ studies (Nicholson et al., 1995) have shown that α is comparable to vertebrates in at least some regions.

The basic similarities in α make the exceptions interesting. Thus, in the rat hippocampus it was reported (McBain et al., 1990) that the volume fraction of CA1 was only 0.12 and the vertical lobe of the cuttlefish may have a similar low value (Nicholson et al., 1995); both these reductions in α may reflect local structural properties. Rice et al. (1993) have recently shown that in the cerebellar molecular layer, when anisotropy is accounted for, the volume fraction reaches 0.31. Previous studies of this region did not

TABLE 26-1. *Recent Values of Volume Fraction (α) and Tortuosity (λ) Obtained with the TMA-Method*

Animal	Region	α	λ	Ref.
Rat	Cerebellum, molecular layer	0.21[a]	1.55[a]	Nicholson and Phillips, 1981
	Neocortex	0.18	1.57	Cserr et al., 1991
	Neocortex	0.18	1.40	Lundbæk and Hansen, 1992
	Neocortex	0.20–0.22	1.63	Lehmenkühler et al., 1993
	Hippocampus, CA1	0.12	1.67	McBain et al., 1986
	Hippocampus, CA3	0.18	1.83	McBain et al., 1986
	Neostriatum	0.21	1.54	Rice and Nicholson, 1991
	Spinal cord, dorsal horn	0.20	1.62	Syková et al., 1994
Guinea pig	Cerebellum, molecular layer	0.28[a]	1.84[a]	Hounsgaard and Nicholson, 1983
Turtle	Cerebellum, molecular layer	0.31	1.44, 1.95, 1.58	Rice et al., 1993
	Cerebellum, granular layer	0.21	1.77	Rice et al., 1993
Skate	Cerebellum, molecular layer	0.24[a]	1.62[a]	Nicholson and Rice, 1986
Cuttlefish	Optical lobe	0.29	1.86	Nicholson et al., 1995
	Vertical lobe	0.10	1.65	Nicholson et al., 1995

[a]These cerebellar measurements in the molecular layer did not take anisotropy into account.

take the anisotropy into account, which is led to errors in estimating α. Indirect evidence from impedance measurements in the retina (Karwoski et al., 1985) suggests that α is 0.12 in the subretinal space and 0.11 in the inner and outer plexiform layers, but only 0.03 in the outer and inner nuclear layers and 0.02 in the ganglion cell and optic nerve fiber layers.

Knowing the volume fraction does not tell us how close the membranes of cells are to each other. Conventional wisdom has it that the spaces are about 15 to 20 nm in width (Schmitt and Samson, 1969), but very recent studies based on the optical imaging of a graded series of fluorescent dextrans indicate that the spaces are larger than 5 nm. A maximum size cannot be given at this time, but there are probably spaces of graded size; however, there are likely some regions less than 15 nm in width because molecules of this diameter are more hindered than smaller entities (Nicholson and Tao, 1993; see next section). Some morphologists (e.g., Bondareff and Pysh, 1968) have also suggested that the spaces between cells are not uniform but may enlarge into "lakes."

TORTUOSITY

Tortuosity defines the hindrance imposed by obstructions in the extracellular space. It was recognized in a number of earlier studies (e.g., Harris and Burn, 1948; Patlak and Fenstermacher, 1975) but only measured systematically with the advent of the TMA method. Because of this hindrance we refer to an *apparent* diffusion coefficient, D^*, in tissue. We can then define a factor, λ, the tortuosity, which accounts for the hindrance to extracellular diffusion that arises from the obstructions presented by cell membranes. Then tortuosity is conventionally defined by $\lambda = (D/D^*)^{1/2}$, where D is the diffusion coefficient in a free medium. Generally speaking (see Table 26-1), the tortuosity is about 1.6 so that $D^* \approx 0.4 \times D$. This implies a significant reduction in the ability of substances to move in the extracellular space compared to movement in a free solution.

Studies of the cerebellum have recently shown (Rice et al., 1993) that the molecular layer is anisotropic so that there are three values of λ (λ is a tensor), each associated with one of the three axes defined by the geometry. Diffusion is least hindered along the parallel fibers (x-axis) and in the direction normal to the surface (z-axis), but in the axis parallel to the surface and at right angles to the parallel fibers (y-axis), greater obstruction is encountered. The values of tortuosity (Table 26-1) are in the ratios: λ_x: λ_y: λ_z = 1.44:1.95:1.58. In practice this means that a substance released into the BEM of the molecular layer spreads out with an ellipsoidal pattern rather than in the shape of a sphere, so that there is a tendency for preferential spread along the parallel fibers. It may be anticipated that this trend would also be seen in white matter tracts where anisotropic impedance has been measured (Ranck and BeMent, 1965). These data also clarify the diversity seen in previous measurements of cerebellar parameters (Nicholson and Phillips, 1981, Houndsgaard and Nicholson, 1983, Nicholson and Rice, 1986), which were probably due to different orientations of the measuring axes with respect to the cerebellar axes. No such anisotropy is seen in the granular layer of the cerebellum, but the tortuosity is higher than average (Rice et al., 1993) (Table 26-1), presumably due to the dense packing of the tiny granule cells.

Recent studies using optical imaging methods (Nicholson and Tao, 1993) have enabled the study of the diffusion of fluorescent dextrans having molecular weights in the tens of thousands in contrast to the studies with TMA^+, which has a molecular weight of 74.1. These studies (Figure 26-2), on the rat neocortex, have revealed that up to a molecular weight of 10,000, the large molecules are no more hindered than TMA^+, but 40,000 and 70,000 MW dextrans demonstrate a significant increase in tortuosity. These data suggest that, although molecules

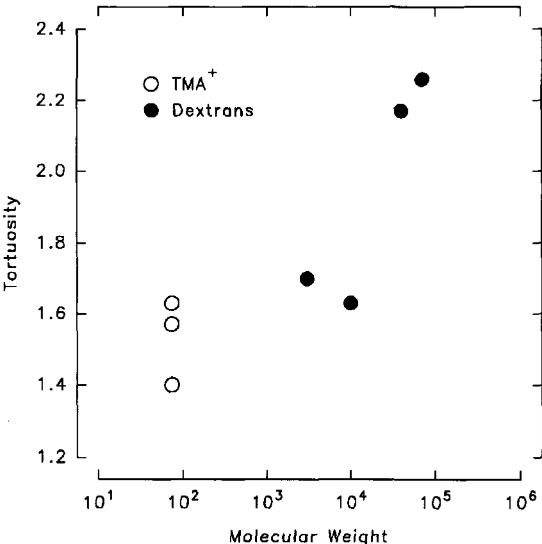

FIG. 26-2. Distribution of tortuosities measured with molecules of different molecular weights in the neocortex of the rat. Abscissa is molecular weight on a logarithmic scale, ordinate is tortuosity. Measurements made with TMA^+ (*open circles*) with molecular weight 74.1 and dextrans with molecular weights 3000 and 10,000 all lie between 1.4 and 1.7. The two largest dextrans of 40,000 and 70,000, however, show tortuosities exceeding 2.0. This suggests that such large molecules are excluded from some regions of the brain extracellular microenvironment.

above a certain size can still diffuse through the BEM, when the molecular weight reaches a value between 10,000 and 40,000 they are excluded from some regions. This is particularly significant, since many diffusible factors of current interest, such as nerve growth factor or its subunits, lie in the range of 26,000 to 130,000 MW (Thoenen and Barde, 1980).

Tortuosity could also reflect constraints that are not strictly geometrical, such as charge interaction with the long-chain tethered proteoglycans and glycosaminoglycans that are found in the extracellular space (Nicholson, 1980; Margolis et al., 1986; Iwata and Carlson, 1993) but so far no clear evidence of charge discrimination has been found (Nicholson and Phillips, 1981, Nicholson and Rice, 1987).

MODEL OF DIFFUSION OVER EXTENDED DISTANCES

The most basic process for the movement of substances through the extracellular space is diffusion, indeed, diffusion is an inescapable component of any transport process. It is also through the analysis of diffusion that volume fraction and tortuosity may be better appreciated. The model described below applies over distance scales of 1 μm or more; in the next section a model for a much shorter scale will be discussed.

A molecule, characterized by a free diffusion coefficient D ($cm^2 \cdot s^{-1}$) in aqueous solution, distributes in the BEM according to a generalization (Nicholson and Phillips, 1981; Nicholson, 1992) of the classic diffusion equation (see Carslaw and Jaeger, 1959 or Crank, 1975 for an extensive treatment of the diffusion equation):

$$\frac{\partial C}{\partial t} = \frac{D}{\lambda^2} \nabla^2 C + \frac{Q}{\alpha} - \frac{F(C)}{\alpha}. \tag{1}$$

The generalization introduces the two nondimensional factors, λ, the tortuosity, and α, the volume fraction, that incorporate the structure of the tissue, as described above. In a free aqueous medium $\alpha = 1$ and $\lambda = 1$, while in brain $\alpha < 1$ and $\lambda > 1$. Time (s) is denoted by t, while ∇^2 represents the second spatial derivative and C is the concentration (mM $\equiv$ $mmole \cdot L^{-1}$) of the diffusing molecules. A source-term Q ($mM \cdot s^{-1}$) describes local release of molecules. Uptake of material from the extracellular space, typically into cells, is defined by $F(C)$ ($mM \cdot s^{-1}$). If this function is zero or a linear function of C then Equation (1) can be solved for a variety of cases (Nicholson, 1992).

Diffusion from a Spherical Cell

To illustrate some of the implication of Equation (1), the equation can be simplified to represent the steady-state diffusion from a spherical cell of radius a. In this problem there are no sources distributed in the tissue, but instead we assume a substance being released across the cell membrane at a constant rate. This substance is generated in the cell at a rate of q μmoles per unit volume per second ($\mu M \cdot s^{-1}$) This means that the total amount leaving the cell is (*cell volume*) $\times$ q per unit time. The substance is removed throughout the BEM with a constant uptake ν ($\mu M \cdot s^{-1}$). This type of uptake is saturable, nonlinear, and an extreme example of Michaelis-Menten kinetics. Equation (1) becomes

$$\frac{D}{\lambda^2 r^2} \frac{d}{dr}\left(r^2 \frac{dC}{dr}\right) - \frac{\nu}{\alpha} = 0, \tag{2}$$

where r is the distance (cm) from the center of the cell and $r \geq a$.

The solution to this problem is more complex (Nicholson, 1995) than it appears at first sight, but intuitively it is clear that near the cell the uptake system is likely to be overwhelmed by the efflux of substance; since the rate of removal is fixed, this means that the uptake saturates. With further distance from the cell, the concentration drops due both to the uptake process and the divergence of the substance as it spreads out in three-dimensional space. As this happens the effectiveness of the uptake increases, and there is accelerated removal of the substance. It is fairly obvious that at some finite and calculable distance, b, all the substance will have been removed. This distance can be shown to be

$$b = a\left(1 + \frac{q}{\nu}\right)^{1/3}. \tag{3}$$

Note that this distance does not depend on the diffusion characteristics of the BEM, it is only a function of the rates of release and uptake per unit volume and the size of the cell. The concentration profile does depend on D, λ, and α and is expressed by

$$C = \frac{\lambda^2}{\alpha D} \frac{\nu}{6} \frac{b^3}{r}\left[\left(\frac{r}{b}\right)^3 - 3\left(\frac{r}{b}\right) + 2\right] \quad \text{for } a \leq r \leq b$$
$$= 0 \quad \text{for } r > b. \tag{4}$$

For comparison, when there is no uptake $\nu = 0$ and Expression 4 becomes

$$C = \frac{\lambda^2}{\alpha D} \frac{q}{3} \frac{a^3}{r}. \tag{5}$$

The consequences of diffusion and uptake are shown in Figure 26-3. Setting the radius of the cell

$a = 5$ μm, and assuming that the diffusing molecule is similar to serotonin (dopamine and a number of other neuroactive compounds have similar diffusion coefficients, see Gerhardt and Adams, 1982). Curves were calculated using Equations (3), (4), and (5) and plotted in Figure 26-3, where the legend further defines the parameter values used in the calculations. With semilogarithmic axes, the curve representing diffusion without uptake falls quite slowly; even at 500 μm the concentration is still 0.02 μM (not illustrated). In contrast, in the case of uptake, the concentration has fallen to zero by 50 μm away from the cell ($r = b$). This simple example illustrates a basic principle. If diffusion is the only process governing molecular migration, then eventually released molecules will spread large distances from the site of release. On the other hand, if uptake (or some form of degradation) is operative, then molecules will not get beyond a certain distance from the source.

LOCAL MODELS OF THE EXTRACELLULAR MICROENVIRONMENT

The interactions between neurons and glia often involve only issues about the influence of a single cell on its neighbor; in this case only very short-range diffusion effects are relevant and the local geometry becomes the dominant issue. The distance scale here would be less than 1 μm.

The classic model of this type is the interaction between the giant axon of the squid and its sheath of Schwann cells, first discussed by Frankenhaeuser and Hodgkin (1956) (Figure 26-1B). The model arose because these investigators noticed that a train of action potentials in the axon were accompanied by undershoots that declined in time. The decline was attributed to accumulation of K^+ adjacent to the axon and this led naturally to models of the environment surrounding the axon. Recent experiments have suggested that in axons in very good condition, the decline in the undershoot is smaller than that seen by Frankenhaeuser and Hodgkin, suggesting that the K^+ is effectively removed by Schwann cells in a variety of ways (Abbott et al., 1991; Brown and Abbott, 1993). Nevertheless the basic model of Frankenhaeuser and Hodgkin is relevant to any discussion of local interactions.

The Frankenhaeuser and Hodgkin Models

The first model consisted of a finite free space of thickness θ (cm) around the axon bounded by a very thin barrier to diffusion with a permeability P ($cm \cdot s^{-1}$). Defining the outflow of K^+ through the excitable membrane of the axon as M ($mmol \cdot cm^{-2} \cdot s^{-1}$), and assuming that there is no gradient in the space, this leads to the simple equation for the concentration C (mM) in the space

$$\theta \frac{dC}{dt} = M - PC. \quad (6)$$

Based on the available electrophysiological and anatomical data, Frankenhaeuser and Hodgkin identified the barrier with the surrounding Schwann cells, which were made permeable by the presence of narrow clefts between the overlapping glia. The space between axon and Schwann cell, θ, was estimated to be 30 nm, and the permeability, P, was estimated to be 6×10^{-5} $cm \cdot s^{-1}$. Recently these estimates were significantly revised by Astion et al. (1988). These authors, working on the small squid *Alloteuthis,* estimated that in extreme cases θ could be as large as 190 nm and P as large as 7×10^{-4} $cm \cdot s^{-1}$.

The second model consisted of a finite diffusion barrier extending directly from the membrane with no intervening space. The barrier was characterized in this model by an apparent diffusion coefficient D^*

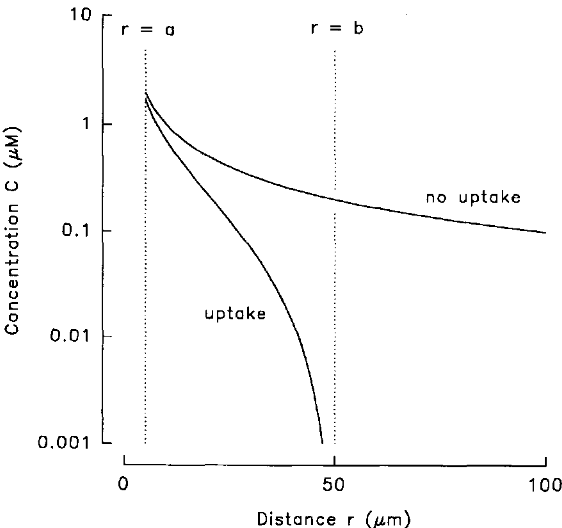

FIG. 26-3. Comparison of effects of simple diffusion and diffusion with uptake on the concentration profile of a small molecule migrating away from a cell in brain tissue in steady-state conditions. Abscissa is distance on a logarithmic scale and ordinate is concentration, on a linear scale. The radius of the cell is 5 μm ($r = a$), the free diffusion coefficient (D) is 5.4×10^{-6} $cm^2 \cdot s^{-1}$ (serotonin at 25°C), $\alpha = 0.2$ and $\lambda = 1.6$. The rate of generation of the substance was taken as 10 $\mu M \cdot s^{-1}$ and the rate of uptake as 0.01 $\mu M \cdot s^{-1}$. While the concentration of the substance falls slowly with distance when there is no uptake, the concentration falls rapidly when uptake is present and no substance migrates further than 50 μm ($r = b$) from the center of the cell. See text for further details.

(Frankenhaeuser and Hodgkin actually called this parameter D but D^* is used here to avoid confusion with the use of D to denote the free solution diffusion coefficient) for the barrier, which had a thickness l. It was assumed that concentration was zero at the outer edge of the barrier. Based on their electrophysiological data, Frankenhaeuser and Hodgkin arrived at values of $D^* = 2 \times 10^{-10}$ $cm^2 \cdot s^{-1}$ and $l = 540$ Å. Since the free diffusion coefficient for K^+ at the temperature of these experiments (18°C) is about 1.5×10^{-5} $cm^2 \cdot s^{-1}$ (Hodgkin and Keynes, 1953), Frankenhaeuser and Hodgkin were actually assuming a tortuosity of 274 for their diffusion layer. One noteworthy aspect of this model is that the barrier did not incorporate a volume fraction, so there was no discussion of how much K^+ was retained in this region.

Frankenhaeuser and Hodgkin found that the first model fitted their data better than the second, which did not predict the electrophysiological data well at early times. Moreover, the first model could be identified with anatomical structure. A more elaborate theoretical evaluation of these diffusion models has been provided by Taylor et al. (1980) (Figure 26-1C) who modeled the space described in Frankenhaeuser and Hodgkin's first model, the clefts between the Schwann cells and subsequent basement membrane, connective tissue, and unstirred layers. Most recently, there has been further interest in assessing the physiological role of the complex Schwann cell morphology and, as it is now appreciated, K^+ transport properties (Abbott et al., 1988, 1991; Astion et al., 1988; Brown et al., 1991; Brown and Abbott, 1993; Lieberman et al., 1993).

The type of models proposed by Frankenhaeuser and Hodgkin have been the basis for other considerations of K^+ homeostasis around cells in the central nervous system. Many fundamental ideas about the diffusion of substances in intercellular clefts and the interactions between neurons and glia were developed by Kuffler and Nicholls (1966), based on studies in the leech. Other examples include Lebovitz (1970), and Adelman and Palti (1972); but all these models were mainly directed to discussions of how accumulation of K^+ affected the electrical properties of cells. With the advent of ion-selective microelectrodes for K^+, other issues came to the fore such as accounting for spatial buffering of K^+. Nevertheless, the sort of models proposed by Frankenhaeuser and Hodgkin and by Taylor and colleagues could be applied to a variety of problems of neuron-glia interaction and, ironically, are probably not well suited to model K^+ because of its ready movement across membranes, but better suited to less permeable compounds.

SPATIAL BUFFERING OF POTASSIUM

The question of whether K^+ constitutes a signal in the BEM is still not totally resolved, but if it does, then glia undoubtedly play a role in controlling this communication channel. Many studies with ion-selective microelectrodes have shown that virtually all neuronal activity is accompanied by an increase in $[K^+]_0$. The normal baseline $[K^+]_0$ is generally taken as 3 mM, but two recent reports (Moghaddam and Adams, 1987; Coles and Poulain, 1991) have suggested that this could be an overestimate and that regional variations exist with values as low as 1.9 mM. If these measurements are correct, then $[K^+]_0$ does not simply reflect the concentration in the cerebrospinal fluid but is locally regulated, and this could involve glial cells. Leaving this interesting issue aside, it is well established that during synchronous activation of neurons, by electrical stimulation, for example, $[K^+]_0$ can rise by several mM and may even go as high as 12 mM, at which level a "ceiling" occurs that is only exceeded in the developing brain and in pathophysiologies such as anoxia and spreading depression. Reviews of studies of evoked changes in $[K^+]_0$ can be found in Walz and Hertz (1983) and Syková (1992).

Both neurons and glia respond to local increases in K^+ by depolarizing because the basic determinant of the membrane potential of all brain cells is the ratio of K^+ across the membrane. Thus the possibility of a widespread and effective signaling mechanism exits. If there is a fundamental flaw in this scenario it is the lack of specific receptors for K^+. All cells respond, so either there is a nonspecific depolarization of a population of cells, which may be functionally relevant, or the BEM has properties that channel K^+ to some cells and not others. These issues raise the question of how $[K^+]_0$ is controlled.

As first pointed out by Orkand et al. (1966), the fact that glial cells have membranes that apparently exhibit a very high selectivity for K^+ means that a special type of mechanism can be brought into play to disperse local accumulation of K^+. This *spatial buffering mechanism* has been analyzed in theory by Gardner-Medwin (1983b, 1986) and by Odette and Newman (1988) and Dietzel et al. (1989). The most extensive experimental evidence for spatial buffering has been obtained in the the retina (Gardner-Medwin et al., 1981; Coles and Orkand, 1983; Newman, 1986; Reichenbach, 1991; Oakley et al., 1992). The mechanism will be elaborated below, but suffice it to say that it depends solely on basic biophysical properties of membranes. An alternative mechanism for control of K^+ is that of energy-dependent transport, the best known example of which is the ubiquitous

oubain-sensitive Na^+-K^+ transporter that moves Na^+ out of cells and K^+ in (Ballanyi et al., 1987; see Chapter 16, this volume). Other transport mechanisms, such as ones that control pH (Chesler, 1990; Jendelová and Syková, 1991), can also move K^+, and passive accumulation of KCl is also possible (Gardner-Medwin, 1980; Ballanyi et al., 1987; see Chapter 18, this volume).

The relative merits of spatial buffering versus active transport have been debated extensively over the years. Useful accounts can be found in Cserr (1986), Barres et al. (1990), and Barres (1991). Barres and coworkers have tended to emphasize the importance of uptake compared to spatial buffering. These issues are addressed in several other chapters in this book (see Chapters 18, 31, 47). With the present emphasis on molecular mechanisms, transporters are likely to receive increasing attention in the years to come, and therefore this chapter discusses spatial buffering in some detail, since, like diffusion, it is a consequence of basic physical principles and therefore is always present, even if the brain has evolved more effective mechanisms to bypass it.

Mechanism of Spatial Buffering

The fundamental concept of spatial buffering is simple (Figure 26-4). If an elongated cell satisfies two conditions—i.e., (1) It is predominantly permeable to K^+ over other ions, and (2) It is bathed in a gradient of K^+ concentration—then the part of the cell

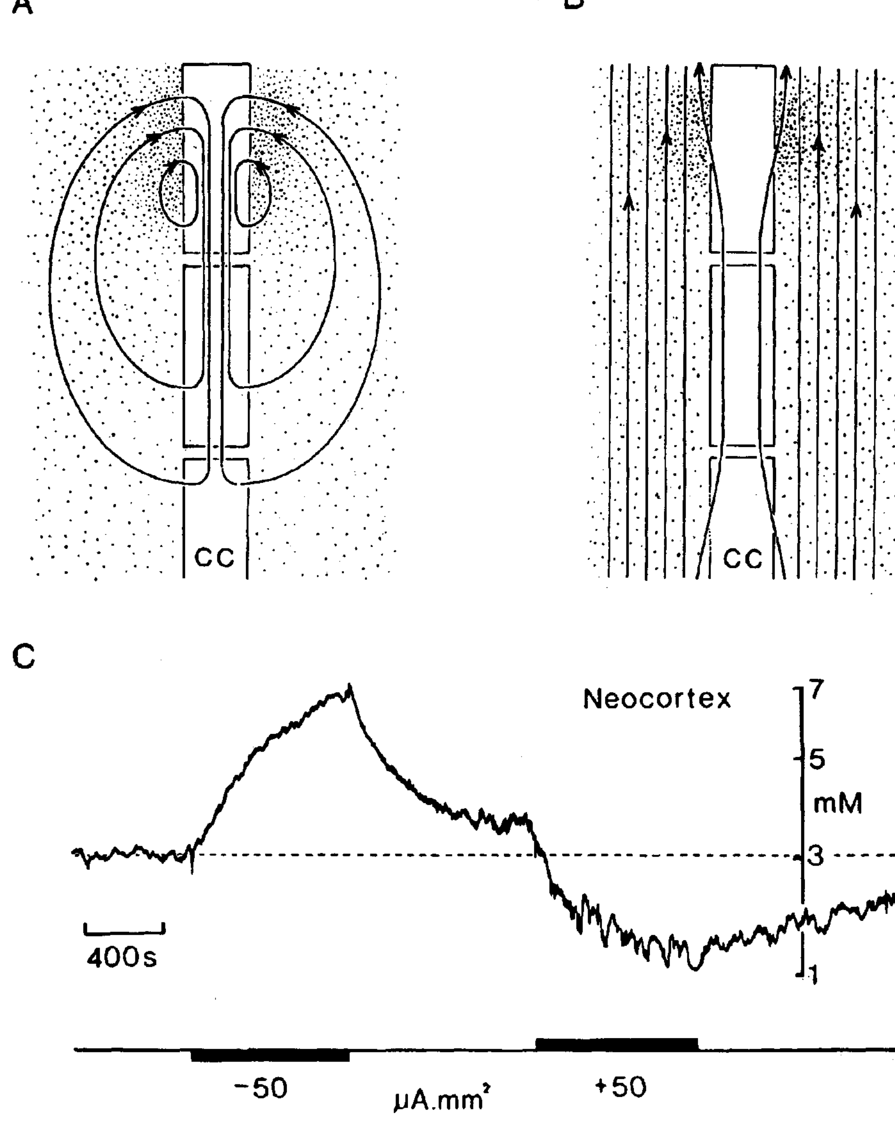

FIG. 26-4. Current-mediated spatial buffering of K^+. (A) A set of vertically oriented, electrotonically coupled core conductors (*cc*) or "transfer cells" that are subjected to a localized increase in $[K^+]_o$ (*density of dots*) over the upper cell surface. The gradient of $[K^+]_o$ causes the upper regions of the core conductor to be more depolarized than the lower, so that a current enters the cell in the upper region and exits over the lower surface in a distribution determined by the cable properties of the cells. Since the membranes of the cells are predominantly permeable to K^+, the current is carried by K^+, and consequently the circulating current rapidly dissipates the $[K^+]_o$ gradient. (B) The converse situation to that shown in Figure A. A vertically flowing current is imposed on a tissue containing vertical core conductors. Much of the current flows through the extracellular space, but some enters the cells in the lower regions and exits in the upper (the proportion and distribution are governed by the cable properties and various conductivities involved). Again, assuming that the membranes are predominantly permeable to K^+, the current carries this ion into the cell in depth and out of the cell superficially, thereby depleting and augmenting $[K^+]_o$, respectively. (C) An experiment in the rat neocortex to verify the concepts diagrammed in Figure B. A current density of 50 $\mu A \cdot mm^{-2}$ is applied, with the brain surface negative and $[K^+]_o$ recorded just beneath the surface. $[K^+]_o$ slowly rises by several mM. When the current is reversed so that the surface is made positive, $[K^+]_o$ falls. [From Nicholson (1983), with permission.]

that is bathed in the higher K^+ concentration will be more depolarized than the other part that is in the lower concentration. Current will flow into the region that is more depolarized and out of the other region, but, since the membrane is dominated by potassium permeability, the current will be carried by this ion, rather than Na^+ or Cl^-. Consequently, K^+ will enter the cell where it is depolarized, which happens to be where the extracellular K^+ is highest, and K^+ will leave where the extracellular K^+ is lowest, thus the gradient of K^+ surrounding the cell will be dissipated. If the permeability of the membrane to K^+ is sufficiently high, then the dissipation process can be very rapid, since it is mediated by electric current flow, and exchange with intracellular ions rather than to actual movement of ions over large distances. Glial cells do indeed appear to have the requisite K^+ permeability, but recent findings suggest that the channels are all voltage-sensitive inward rectifiers (Barres et al., 1990), which may imply that K^+ enters cells more easily than it leaves. A second problem, recognized early in the theory, is that the short length constant of glial cells and short physical length might preclude movement of K^+ over significant distances; however, the evidence for extensive coupling of glial cells is often mentioned as a solution to that problem (see Chapter 19, this volume).

The real issues cannot be dealt with completely by simple intuitive models, however, and it is fortunate that Gardner-Medwin has developed an extensive theoretical framework for the discussion of spatial buffering, which has been little used to date. Given our greatly expanded knowledge of glial cell properties, it would now be worthwhile to reconsider the issues in the light of this model.

Gardner-Medwin has provided three definitive papers (Gardner-Medwin, 1983a, 1983b; Gardner-Medwin and Nicholson, 1983) and a final and important paper (Gardner-Medwin, 1986) extending some of the ideas. Other papers, dealing with implications for pathophysiology are referenced in the four cited papers.

No attempt here will be made to describe in detail the theoretical model of Gardner-Medwin; rather the goal will be to indicate the importance of the results. The papers are based on a combined experimental and theoretical approach. The experiments are based on a clever innovation. If the spatial buffer hypothesis is correct, then it must be possible to "run it backwards." In other words, if the distribution of K^+ in the brain is initially uniform and we apply a steady current so that a *potential* gradient is established, then K^+ will enter glia cells where they are subject to highest potential and leave where the potential is lowest, and this will lead to $[K^+]_0$ depletion in the one region and accumulation in the other. A gradient in extracellular K^+ will therefore be *created*, and this can be measured in a variety of ways (Figure 26-4). This prediction was completely verified in several experiments, and, moreover, shown to be an extremely robust phenomenon that persisted when transport systems were blocked (Gardner-Medwin and Nicholson, 1983), supporting the idea that it is a fundamental biophysical property.

The second part of Gardner-Medwin's work developed a detailed model of the processes involved in both the normal spatial buffer mechanism and when it was reversed. While the ideas are simple at one level, they are complex at another, since it is necessary to combine (1) the coupling of potential gradients with chemical gradients and diffusion and (2) the electrotonic behavior of the leaky distributed structures that represent glia and neurons. Item 1 involves solving the Nernst-Planck equation and item 2 involves the application of cable theory. The combination leads to formidable equations that require numerical solution. It is worth noting that in all his formalism Gardner-Medwin referred to the mediators of spatial buffering as "transfer cells" to avoid categorically identifying them with glia.

Even without a detailed analysis, it is fairly evident that the extent to which a population of glia implement spatial buffering will depend on factors of geometrical distribution. In particular, the relation between the spatial extent of the potassium inhomogeneity and the length constants of the cells involved. This issue of what types of potassium distribution could be effectively dissipated by spatial buffering and which would be more amenable to uptake was the topic of Gardner-Medwin's work (1986), where the concept of spatial frequencies was introduced to characterize distributions.

CONCLUSIONS

There is little doubt that the extracellular space is an effective conduit for the movement of molecules and there is little doubt that both neurons and glia release substances into this space, consequently there must be a continual molecular traffic between cells and cell populations. This flow can be channeled by local architecture of the extracellular space and by mechanisms that remove the chemical messengers from the extracellular space. While we have no clear picture of this control system at present, glial cells, which often exhibit massive membranous expansions, seem very well suited to play a major part in the regulation of molecular traffic in the extracellular microenvironment.

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27 Functional significance of glial-derived matrix during development and regeneration

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In the last decade, our understanding of the role glial cells play in the developmental and regenerative potential of the nervous system has increased dramatically. However, while it is clear that neuroglial cells are involved in neuronal migration (Hatten, 1990; Rakic, 1990), process outgrowth during development (Silver, 1984; Bovolenta and Mason, 1987; Silver et al., 1993), and regenerative failure within the adult central nervous system (CNS) (Reier and Houle, 1988), the molecular mechanisms involved in these events are only recently becoming clear. In this chapter, we review the various roles played by glia, specifically astrocytes, during development with special emphasis on, first, the cell-surface molecules associated with these cells and, second, glycoproteins and proteoglycans secreted by these cells into the extracellular matrix (ECM), which have been suggested to influence axonal outgrowth. Particular emphasis is placed on the molecular changes that occur within these cells once development is complete and the effect these changes have on axonal regeneration. Because this review focuses on the role played by only one type of neuroglial cell, the astrocyte, during vertebrate development and regeneration, the reader is referred to Chapters 57 and 59 in this volume for reviews of the two other major neuroglial cell types, oligodendrocytes and microglia.

HISTORICAL COMMENTS

Glial Structures as Axonal Pathways

The intimate relationship between growing axons and glial cells was perhaps best highlighted in 1928 when Ramón y Cajal documented an association between regenerating axons and glia (Schwann cells) within the sciatic nerve of the peripheral nervous system (Ramón y Cajal, 1928). However, it was not until much later that a similar role played by CNS glia was as clearly demonstrated. Singer et al. (1979) reported that during the development or regeneration of spinal cord axon tracts in the lizard, extracellular spaces between glial cells were present, which seemed to form longitudinal "channels" ahead of the leading axons. It was suggested that these channels may provide a physical and/or chemical pathway for axon growth. Interestingly, elongating axons were always found associated with the glial processes that formed the walls of the channel. Similarly, Silver and Robb (1979) reported the presence of preformed glial channels within the retina of embryonic day 10 mice, 2 days prior to the growth of optic axons out of the eye. Evidence that these channels were involved in axonal growth was provided by studies using the ocular retardation mouse mutant (*orj*) in which the channels between the primitive glial processes are reduced in size to approximately 10% of those found in the normal mouse retina. In this mutant, retinal ganglion cell axons never leave the eye. Instead, large neuromatous whorls of growing axons form at the interface between the retina and the pigmented epithelium. These data suggested that a change in the glial environment in *orj* mutants may have been involved with the failure of optic fibers to grow out of the eye (Silver and Robb, 1979). However, while it had now been suggested that the environment created by glia was important for the development of certain long-fiber projections, it was not clear whether the association between growing axons and glia was simply due to the available space provided by glial channels or whether there was a more active, molecular association between these two cell types.

In 1984 another primitive glial structure that influenced the direction of optic fibers was reported at the boundary between the diencephalon and telencephalon (Silver, 1984). In this region, central projections from optic and olfactory regions converge but remain segregated and project to different areas of the brain. Precisely at the area where these two projections converge, a "knotlike" structure of spe-

cialized neuroepithelium, which eventually differentiate into glia, was discovered. Just prior to the arrival of axons, this area undergoes a significant amount of cell death and subsequent remodeling to form a compacted cellular architecture. When growing fibers reach the "knot" it consists of a dense cluster of interwoven nonneuronal cells that lack marginal radial processes and whose cell bodies directly abut the glial limiting membrane. The morphology of this area is in sharp contrast to the channelized marginal zone and lengthy glial endfoot regions along which axons tend to grow normally. It was suggested that, due to the absence of large extracellular spaces and the elimination of the marginal zone processes, the specialized glia within the diencephalic-telencephalic junction may serve as an axon refractory area, or boundary, which served to separate the optic and olfactory projections.

Another example of the interaction between growing axons and glial structures occurs during the development of the corpus callosum. As the fibers within the callosum project toward the midline, they are faced with a number of decisions. At the corticoseptal interface, callosal fibers continue across the midline in order to terminate in the opposite hemisphere, thereby passing up another potential target, the septum. Present in this area is a unique glial structure, which may help influence this choice. This structure has been described as a glial "sling," which develops at the midline during the growth of the first callosal axons (Silver et al., 1982). This "sling" consists partly of radial astroglia and partly of a dense mat of primitive subventricular cells that is located dorsal to the partially fused septum. Because the initial callosal axons migrate along the dorsal surface of this structure in order to cross the midline, it has been proposed that the glial "sling" specifically functions to guide callosal axons across the midline at the point where the two cerebral hemispheres begin to fuse (Silver et al., 1982).

While it has been suggested that the glial "sling" is involved in guiding the initial callosal axons, the region of the "sling" is only 200 to 250 μm long. However, by the time its development is complete, the corpus callosum will have expanded rostrally and be approximately 2 mm wide. Thus, while the glial sling may be involved in the initial stages of callosal development, other mechanisms must contribute to the formation of the rest of this projection. Specifically, axon fasciculation and specialized glia that are associated with the continued fusion of the telencephalic midline in a rostral direction (Hankin and Silver, 1986) leads development of the callosal commissure (Silver et al., 1993).

In summary, these studies served to reinforce the concept of an *in vivo* axonal/glial interaction that helps to determine the extent and direction of axon outgrowth. However, axons can be directed in two ways. First, astroglia may provide preferred pathways for axon outgrowth or, second, they may serve as barriers in precise locations to prevent the growth of certain axon pathways into inappropriate areas.

ROLE OF CELL AND SUBSTRATE ADHESION MOLECULES IN AXON-GLIA INTERACTIONS

Cell Adhesion Molecules

Up to this point, we have discussed glial structures that may direct or segregate growing axon pathways. This is not to imply, however, that axons interact with glia due strictly to the location or density of specialized subpopulations of these nonneuronal cells. Rather, it is now known that, *in vitro,* axons actually prefer to grow on (Noble et al., 1984; Fallon, 1985), or specifically avoid (Grierson et al., 1990), particular types of glial cells. Attractive versus repulsive glial surfaces have been most clearly demonstrated when axons are given a choice between different cell types (Noble et al., 1984, Fallon, 1985). In cases where glial cells form an attractive surface, it was suggested that this association was due to the expression of specific cell and substrate adhesive molecules, including the neural cell adhesion molecule (NCAM) (Noble et al., 1985), N-cadherin (Neugebauer et al., 1988; Tomaselli et al., 1988), and laminin (Liesi et al., 1984).

NCAM. *In vivo,* NCAM has been shown to be associated at the endfoot region of primitive glia and on the marginal radial process of neuroepithelial cells in areas of the optic pathway (Silver and Rutishauser, 1984). It is in this NCAM-positive area that most early growth cones are found and is thus consistent with the marginal location of the pioneering optic fibers (Silver, 1984). Additionally, when anti-NCAM Fab's are injected into the early developing eye cup of chicks, the optic fibers dislocate from the glial limitans as they exit the eye (Silver and Rutishauser, 1984). These data are consistent with the proposed interaction between axon outgrowth and NCAM-producing neuroepithelial cells within the retina. Finally, neuroepithelial cells within the glial "knot" (described above) do not express NCAM during the time of initial axon outgrowth nor later in development (Silver et al., 1987). Along with the morphological changes in this region, the absence of NCAM may contribute to the glial "knot's" function as an axon-refractory area (Silver et al., 1987).

N-Cadherin. Another molecule involved in promoting neurite outgrowth on glia is N-cadherin (Neugebauer et al., 1988; Tomaselli et al., 1988), which is one member of a family of Ca^{+2}-dependent cell adhesion molecules. N-cadherin appears to be the predominant cadherin in the CNS and has been implicated in neurite outgrowth, since it serves as a potent substrate for the growth of ciliary ganglion neurons in a manner that differs from laminin (Bixby and Zhang, 1990). N-cadherin is present on both neurons and glia (Hatta et al., 1987) where it appears to function via a homophilic binding mechanism. N-cadherin helps promote neurite outgrowth on substrates of astrocytes (Neugebauer et al., 1988) or Müller cells (Drazba and Lemmon, 1990). Finally, N-cadherin may be involved in axon fasciculation, since antibodies to N-cadherin caused a significant change in the morphology of retinal ganglion cell axons, without a change in the amount of outgrowth, when grown on a substrate of purified laminin (Drazba and Lemmon, 1990).

Glia-Derived Nexin. To this point, we have discussed the role of glial-associated molecules that promote neurite outgrowth by providing an adhesive substrate for growing neuronal processes. However, other mechanisms that can promote neurite outgrowth by regulating the degradation of the ECM by protease inhibitors have been described (Monard, 1990). One of the best characterized protease inhibitors is the glia-derived nexin (GDN). This 43 kD protein, present in medium conditioned by glioma cells or primary astrocytic cultures, serves as both a neurite-promoting factor for a variety of cell types and a specific inhibitor of serine proteases, such as thrombin and trypsin (Farmer et al., 1990). Recent evidence has demonstrated that the protease inhibitory activity of GDN is crucial for promoting neurite outgrowth from hippocampal neurons (Farmer et al., 1990). GDN is abundant on the surface of astrocytes and also binds to the ECM, possibly by a heparinlike molecule (Halfter et al., 1989). In adult animals, GDN is most highly expressed in the olfactory system, where it has been suggested to play a role in the continued growth of olfactory neurons through adulthood by interacting with cell adhesion molecules, that is, L1 and/or N-CAM, and possibly laminin to promote neurite outgrowth from these cells (Doucette, 1990). In fact, the interaction between protease inhibitors present in astroglial-conditioned media and laminin have been suggested to be necessary for prolonged neurite outgrowth (Shea et al., 1992). In this model, the rapid elaboration of unstable neurites has been shown to be due to the action of glial-derived protease inhibitors. This rapid neuritogenesis is then thought to be stabilized by ECM components, specifically laminin, allowing for the continued elongation of growing neurites (Shea et al., 1992).

Substrate Adhesive Molecules

Laminin. In addition to the role of cell surface adhesion molecules, substrate adhesive molecules produced by glia and secreted into the extracellular matrix have also been implicated in axonal guidance (Cohen et al., 1986; Liesi and Silver, 1988). Punctate deposits of laminin have been localized near the endfeet of neuroepithelial cells in the developing mouse (Liesi and Silver, 1988) and chick optic system prior to the arrival of the first optic axons (Halfter et al., 1983). The location of these deposits within developing axon tracts is consistent with the *in vitro* observation that glial cells are capable of producing laminin and promoting robust axon outgrowth. However, similar to the expression of NCAM, laminin is only present within developing axon tracts, in close approximation to growing axons, at early stages. As the optic system matures, laminin expression decreases (Cohen et al., 1986; Liesi and Silver, 1988), and molecules involved in axon fasciculation, such as L1 (Lagenaur and Lemmon, 1987), may take on a more important role (Schlosshauer and Dutting, 1991). Consistent with its presence on glia within the optic system, laminin is also associated with glia during the development of the corpus callosum, fornix, and pathways in the embryonic hindbrain (Liesi and Silver, 1988). Finally, Smith et al. (1986) demonstrated that immature activated glia are capable of depositing laminin onto a Millipore filter in a model of axon guidance of the corpus callosum. Since laminin is an extremely potent factor for neurite outgrowth *in vitro* (Liesi et al., 1984), its location on astrocytes during development suggests that it may also serve as a growth-promoting factor *in vivo*.

Molecular Aspects of Immature Glia

Effect of Antibody Perturbations on Axon Growth on Astrocytic Substrates. Because of *in vitro* and *in vivo* studies on the localization of both cell and substrate adhesive molecules during development, subsequent experiments sought to clarify the function of molecules on the surface of astrocytes thought to be involved in promoting neurite outgrowth. Previous studies had demonstrated the presence of laminin (Liesi et al., 1983) and the cell adhesion molecules NCAM and N-cadherin (Neugebauer et al., 1988) on astrocytes. In order to determine whether these adhesion molecules were involved in axon out-

growth, recent studies (Tomaselli et al., 1988; Smith et al., 1990) utilizing function-blocking antibodies to several of these molecules (i.e., N-cadherin, NCAM, and L1), or their receptors (i.e., β1-class integrin), have demonstrated that neurite outgrowth on an astrocytic substrate is decreased in the presence of these reagents. However, in order to achieve the maximal inhibition, a number of different antibodies were required simultaneously. These studies suggested, first, that these molecules are directly involved in promoting neurite outgrowth on astrocytes *in vitro,* but, second, that neurites use more than one type of molecule or mechanism to extend across an astrocytic surface. Unfortunately, whether these same molecules and their receptors are equally capable of promoting axon outgrowth *in vivo* is still not known.

INHIBITORY MATRIX MOLECULES ASSOCIATED WITH ASTROGLIA IN BOUNDARY AREAS DURING DEVELOPMENT

While astroglia may promote neurite outgrowth through the expression of various cell and substrate adhesive molecules, certain subpopulations of astrocytes may serve as barriers to the advance of axons and thereby influence the direction of process outgrowth via a repulsive mechanism. In this regard, during development astrocytes have been shown to secrete certain molecules into the surrounding ECM that can actively repel axon growth cones. One class of putative inhibitory matrix molecules associated with astrocytes are the proteoglycans (PGs). Proteoglycans consist of a protein core to which are attached various glycosaminoglycan side chains (GAG) (Margolis and Margolis, 1989). Proteoglycans are usually classified by the composition of these GAG side chains and consist of chondroitin sulfate, dermatan sulfate, heparan sulfate, and keratan sulfate (Margolis and Margolis, 1989). Due to their size and the heterogeneous nature of these side chains on a particular core protein, the specific composition of proteoglycans can be quite diverse (Herndon and Lander, 1990). However, it is clear that a number of different forms are present in the nervous system (Herndon and Lander, 1990), are synthesized by both neurons and glia (Gallo and Bertolotto, 1990; Flaccus et al., 1991; Johnson-Green et al., 1991), and the different forms can have different functions (Chiu et al., 1986; Snow et al., 1990a, 1990b). As an example of this diversity, it is known that heparan sulfate PG can interact with laminin to form a complex that promotes neurite outgrowth (Dow et al., 1991). Alternatively, certain keratan sulfate PGs (Cole and McCabe, 1991; Geisert and Bidanset, 1993) and chondroitin sulfate PGs (CS-PG) (Snow et al., 1990b) have been shown to inhibit neurite outgrowth when used as a substrate in culture. Initial work in our laboratory with a cartilaginous CS-PG has demonstrated that the GAG side chains were responsible for inhibiting neurite outgrowth *in vitro* (Snow et al., 1990b), although the protein core has also been implicated (Oohira et al., 1991).

Since CS-PG is capable of restricting neurite outgrowth *in vitro,* might this molecule be involved in forming barriers to growing axons during development? Recently, our laboratory, as well as others (Steindler et al., 1989; Cole and McCabe, 1991; Geisert et al., 1992), have examined the expression of these inhibitory molecules in a number of areas normally refractory to axon outgrowth during development.

The Roof Plate

As described above, structures composed of specialized nonneuronal cells are often located in regions of the CNS where they may serve as barriers to growing axons. These barriers may restrict axon outgrowth simply due to their physical properties, as has been suggested to occur during scar formation (Reier and Houle, 1988), or they may actively direct growing axons to other regions by expressing specific growth inhibitory molecules on their cell surface or in the surrounding ECM (Steindler et al., 1989). One area that has been closely examined is the roof plate of the developing spinal cord (Snow et al., 1990a). Located along the dorsal midline, the roof plate consists of glial cells that undergo a morphologic change between embryonic days 12.5 and 15.5. At this earlier time point, the roof plate glia are organized into a wedge-shaped structure, with large extracellular spaces between the cell apices. By embryonic day 15.5, the cells in this area have become reorganized into a dense midline septum, stretching from the pia mater to the central canal. Concomitant with this morphologic change, the roof plate glia begin to express a keratan sulfate-chondroitin sulfate proteoglycan (KS/CS-PG), a molecule unique to these cells. The expression of this molecule is coincident with the growth of axons along, but never across, the dorsal midline. The expression of this molecule is consistent with the observation that KS/CS-PG can inhibit neurite outgrowth *in vitro* (Snow et al., 1990b; Geisert and Bidanset, 1993). These data suggest that the expression of this, and potentially other, inhibitory molecules to axon outgrowth may form not only a physical barrier but also a molecular barrier to growing axons.

The barrier function of the roof plate glia has been further examined in the developing optic projection to the hamster tectum (Snow et al., 1990a; Wu et al., 1990). By creating a carefully placed lesion in the roof plate prior to axonal elongation in this area, Wu et al. (1990) were able to show that aberrant optic projections would cross through the lesioned area and expand into the denervated tectum, a situation that does not occur in normal nor sham-lesioned control animals. Similar to the expression seen in the rat, KS/CS-PG was also localized to the roof plate in the hamster tectum (Snow et al., 1990a; Wu et al., 1991) where it may be involved in preventing elongating axons from expanding across the midline and into inappropriate target areas.

Olfactory Bulb

Recently, it has been suggested that astroglia may be involved in the development of olfactory glomeruli, unique morphologic structures within the olfactory lobe (Gonzalez and Silver, 1993). The olfactory glomeruli are unique because in this area axons form exceptionally tight bundles among the primary dendrites of mitral cells located in the olfactory bulb (Graziadei and Monti-Graziadei, 1980). Although olfactory axons are capable of regenerating throughout life, they never penetrate deeply into the bulb, consistently terminating within the glomeruli (Graziadei and Monti-Graziadei, 1980). In the olfactory bulb of the moth, *Manduca sexta,* Tolbert and Oland (1990) have suggested that glia are involved in glomerular formation, since glial elements define the periphery of the glomeruli for primary olfactory axons and the dendrites of the second-order neurons. Similarly, in the rat olfactory bulb, Bailey and Shipley (1992) have described a number of astrocytic subtypes in the different layers of the bulb and a highly specific relationship with the glomerulus. Finally, Valverde et al. (1992) have suggested that it is the interactions between olfactory axons and bulb glia that initiated the formation of the glomeruli.

In order to examine the molecules involved during the formation of the glomeruli, Gonzalez et al., (1993) recently compared the expression of a specific label for growing olfactory axons, olfactory marker protein, with markers for both immature and mature astroglia, the astroglia-associated ECM glycoconjugate, CS-PG, and cytotactin/tenascin (CT). Cytotactin/tenascin was examined because of its close association with CS-PG (Crossin et al., 1989) and because previous studies have demonstrated that this molecule is involved in the formation of glial barriers within the cerebral cortex (Crossin et al., 1989; Steindler et al., 1989).

The results of these experiments demonstrated that both CS-PG and CT are associated with astrocytes prior to formation of the glomeruli. During later developmental stages, the expression of these molecules changes as astroglia migrate into regions surrounding the developing glomerulus. Now, rather than being somewhat uniformly expressed within the olfactory bulb, CS-PG and CT are expressed on astrocytes which form a ring around the glomerular border. Olfactory axons fail to grow beyond the glomerulus once the increased expression of these molecules has occurred. These data suggest that these molecules are involved in the formation of a barrier to ingrowing axons both during development and throughout the life of the animal.

Retina

To this point, we have described studies that suggest that certain extracellular matrix molecules associated with glia are involved in axonal pattern formation within the CNS. Additional evidence from our laboratory, using the developing rat retina as a model, has demonstrated that these same molecules may also have a role in controlling retinal ganglion cell differentiation (Brittis et al., 1992) as well as the direction of the growth of their processes (Snow et al., 1991). On embryonic day 12, CS-PG is present throughout the retina. By embryonic day 13, CS-PG disappears from the center of the retina, just dorsal to the optic fissure. The CS-PG negative area is the first to become immunoreactive for the neuronal marker, TUJ1, which labels an isoform of β-tubulin found only in cells generating an axon. By embryonic day 14, the area of the retina positive for CS-PG has receded to the periphery, while the area in the center, positive for TUJ1, has expanded into areas previously CS-PG positive. By embryonic day 16, CS-PG staining is restricted to the dorsal and ventral poles of the retina, while TUJ1-positive axons of the retinal ganglion cells are present throughout the retina. Interestingly, retinal ganglion cells at the interface between the receding wave of CS-PG and the expanding zone of TUJ1-positive axons remain in an axonless, primitive state. Only as CS-PG expression continues to recede do these cells begin to put out axonal processes. Therefore, it has been hypothesized that neurons differentiate and extend axons toward the optic fissure in the center of the retina, at least in part, because the expression of CS-PG remains highest at the periphery. This possibility received support from additional experiments using chondroitinase ABC to remove the CS GAG side chains from the protein core (Brittis et al., 1992).

Retinas allowed to develop *in situ* in the presence of this enzyme demonstrated both ectopically placed retinal ganglion cell bodies and a severely perturbed distribution of ganglion cell axons, including some directed away from the optic fissure. Taken together, these data suggest that a receding wave of CS-PG within the retina may help control the timing of retinal ganglion cell differentiation and the direction of axon outgrowth by limiting the direction developing axons can grow.

Barrel Fields

Another glial-associated matrix molecule that may play a role in the formation of barriers is cytotactin-tenascin (Crossin et al., 1989; Steindler et al., 1989). CT is produced by astrocytes (Grumet et al., 1985) and secreted into the ECM where it is often found associated with CS-PG (Crossin et al., 1989). As mentioned earlier, CT is expressed in proposed barrier regions, such as the glomeruli of the olfactory bulb (Gonzalez et al., 1993) and also in regions of glial scarring following cortical (McKeon et al., 1991) and cerebellar injury (Laywell et al., 1992). Additionally, it has been demonstrated that both PNA-lectin binding glycoconjugates and CT are associated with astrocytes in boundary regions of somatosensory cortical whisker barrels (Steindler et al., 1989; Crossin et al., 1989). Steindler et al. (1989) have suggested that these molecules may be involved in the formation of functionally segregated domains within the somatosensory cortex, thereby preventing neurites from crossing into functionally distinct domains. This hypothesis has received support from experiments which examined barrel formation following whisker ablation and demonstrated a shift in the barrier formation of the barrel fields, consistent with the extent of whisker ablation (Crossin et al., 1989).

Additional evidence that CT can serve as an inhibitory molecule comes from *in vitro* studies which have demonstrated that CT can limit cell migration (Crossin et al., 1989) and, when used as a substrate in a choice assay, is inhibitory to neurite outgrowth (Faissner and Kruse, 1990). *In vitro,* subpopulations of astrocytes express CT on their surface (Grierson et al., 1990). When neurite outgrowth on cultured astrocytes was examined, it was observed that neurites grew only on CT-negative astrocytes while avoiding those astrocytes that expressed CT (Grierson et al., 1990). Treating confluent astrocytic cultures with fibroblast growth factor (FGF) increased the percentage of astrocytes that were CT-positive, with a concomitant decrease in the amount of neurite outgrowth on this substrate (Petroski et al., 1991).

Although there is evidence to suggest that CT can serve as an inhibitory molecule, there is additional evidence to suggest that it can also serve as an adhesion molecule (Locher et al., 1991). Recent evidence has suggested that CT contains both cell-adhesive and -inhibitory domains (Prieto et al., 1992; Chapter 28, this volume). The response of any particular cell type to CT may depend on its interaction with a specific domain of this multifunctional molecule. For additional details regarding the possible functions of this complex molecule the reader is referred to Chapter 28.

CHANGING ROLE OF ASTROCYTES DURING DEVELOPMENT AND REGENERATIVE FAILURE

From the studies cited above, it is now clear that astrocytes can promote neurite outgrowth both *in vivo* during development (Silver, 1984) and *in vitro* (Noble et al., 1984; Fallon, 1985), and that subpopulations of astrocytes in discrete regions are involved in forming barriers during development (Silver, 1984; Silver et al., 1993). It is equally well established that astrocytes are also involved with regenerative failure within the CNS of mature animals following injury (Reier and Houle, 1988). Consequently, a change in the neurite promoting capabilities of astrocytes must occur as nonamphibian animals mature (Geisert and Stewart, 1991).

In Vivo *Studies*

Evidence to support this hypothesis comes from studies utilizing the corpus callosum as a model for regeneration. Smith et al. (1986) lesioned the corpus callosum in neonatal mice, prior to the complete formation of this pathway. At the same time they implanted a piece of Millipore filter paper into the lesioned cavity and assessed whether the callosum would continue to develop. Microscopic analysis revealed that a layer of intensely GFAP-positive astrocytes had migrated onto and now covered the implant. Although apparently reactive, these immature astrocytes were capable of allowing neurite outgrowth, since a large number of axonal processes were seen in close association with these cells. In contrast, when implants were placed into the lesion cavity of older animals, no neurite outgrowth was seen across the implant. Again, intensely GFAP-positive astrocytes were present on the implant as seen in younger animals, but these seemed incapable of supporting neurite outgrowth. This observation

was further extended when cultures of purified astrocytes were grown on Millipore implants prior to implantation. Astrocytes derived from neonatal cortex and maintained in culture for less than 1 week were capable of supporting neurite outgrowth even when implanted into the lesioned callosum of adult animals. Astrocytes allowed to "age" in culture longer than 1 week were not able to support neurite outgrowth and again resulted in a lack of axon extension along the surface of the implant (Smith et al., 1986). These data suggested that a critical period existed for the neurite-promoting abilities of astrocytes. Prior to postnatal day 8, astrocytes were capable of promoting robust neurite outgrowth even in the callosum of adult animals where regenerative failure usually occurs. After this time point, it was hypothesized that changes occurred as the astrocyte matured that made it more refractory to axon outgrowth.

Explant Studies of the Glial Scar

The hypothesis that astrocytes become a less conducive substrate for neurite outgrowth after the critical period was further extended by studies that utilized an *in vitro* approach to analyze neurite inhibitory factors involved in glial scarring (Rudge and Silver, 1990). A piece of Millipore was placed into the gray matter of the cerebral cortex in neonatal or adult animals. After time points ranging from 6 to 10 days, the implants were removed, placed in culture, and used as a substrate for embryonic hippocampal neurons. When neurite length was assessed, significantly longer neurites grew on the surface of implants removed from neonatal as opposed to adult animals. This was not attributed to the presence of neuronotoxic substances or inaccessibility of the astrocyte surface. Rather, it was suggested that the inability of glial tissue removed from an adult animal to support neurite outgrowth was due to the expression of molecules on the surface of the astrocyte that inhibited axon outgrowth either directly or indirectly by occluding growth promoting factors found either on the surface of the astrocyte or within the surrounding extracellular matrix (Rudge and Silver, 1990).

Molecular Aspects of Mature Glia

While it was apparent that astrocytes removed from mature animals were not as capable of supporting neurite outgrowth as those obtained from neonatal animals, the molecules involved in this change had not been identified. As previously mentioned, antibodies to NCAM, N-cadherin, and the integrin β1 laminin receptor have been used to demonstrate the role these molecules play in neurite growth on substrates of immature astrocytes (Tomaselli et al., 1988; Smith et al., 1990). In order to determine whether a change in the expression of these molecules was involved in the decreased ability of mature astrocytes to support neurite outgrowth, astrocytes were first allowed to "age" in culture (Smith et al., 1990). Neurite outgrowth on these aged astrocytes was then assessed in the presence or absence of antibodies to these adhesion molecules or their receptors. Interestingly, the reduction in the amount of neurite outgrowth on "mature" astrocytes in the presence of these antibodies was significantly less than the reduction seen on immature astrocytes (Smith et al., 1990). These data suggested that a decreased expression of these molecules on mature versus immature astocytes may underlie the changes seen in the ability of astrocytes to support neurite outgrowth over time. However, while neurite outgrowth can be decreased by antibody perturbations of adhesive molecules or their receptors, the amount of neurite outgrowth that occurs on cultured astrocytes, both immature and "aged," is still significantly greater than that seen on glial scars removed *in toto* from the cerebral cortex of adult animals (Rudge and Silver, 1990). This suggests that there are other factors present *in vivo* which further modify the glial scar and make it an even more nonpermissive substrate for neurite outgrowth.

REEXPRESSION OF INHIBITORY MATRIX MOLECULES IN SUBPOPULATIONS OF REACTIVE GLIA AFTER INJURY

Since previous studies *in vivo* have demonstrated that glia-associated proteoglycans are present in areas of the developing CNS that are nonpermissive for axon outgrowth, we decided to examine whether these same putative inhibitory molecules were, first, present in an area of glial scarring and, secondly, whether those molecules associated with reactive glia were capable of inhibiting growing neurites.

Expression of Inhibitory Molecules Following Injury

In order to examine the expression of both adhesive and putative inhibitory matrix molecules after injury, we again utilized the filter implant paradigm described above (McKeon et al., 1991). When placed into the cerebral cortex of neonatal rats, Millipore filters elicited an increase in the number of GFAP-positive astrocytes. This increase was appar-

ent after 6 days and persisted for at least 30 days. These reactive astrocytes were present within the pores of the filter implant as well as on the surface and within the parenchyma of the brain surrounding the implant. Interestingly, unlike mature astrocytes in culture, increasing amounts of laminin and fibronectin were detected on the surface of the implant over time, consistent with the suggestion that, *in vivo,* a population of astrocytes was capable of maintaining the expression of these potentially growth-promoting extracellular matrix molecules. Finally, the distribution of collagen type IV was examined and determined to be limited to blood vessels growing across the surface of the implant.

When identical filters were implanted into young adult animals the distribution of reactive astrocytes, laminin, fibronectin, and collagen type IV was, surprisingly, similar to that seen in neonatal animals at the same postimplantation time points. Significantly different, however, was the expression of two putative inhibitory extracellular matrix molecules, CT and a CS-PG. Both molecules were found associated with reactive astrocytes either on the surface or within the pores of the filter implant. Interestingly, astrocytes within the surrounding areas of the brain were intensely GFAP-positive, but were CT and CS-PG negative. These data demonstrated that there was a differential expression of putative inhibitory molecules in older versus neonatal animals on a subpopulation of astrocytes intimately associated with the implant. This suggested that only a subpopulation of astrocytes may be functionally reactive, that is, associated with inhibitory molecules, despite the fact that there was a significant increase in the expression of GFAP in a greater number of astocytes surrounding the implant.

The proposed axon inhibitory function of these molecules after direct cortical injury is supported by an *in vivo* model of regenerative failure after indirect spinal cord injury. Prior to, but not after, postnatal day 3, damaged processes of sensory neurons in the dorsal root ganglion can regenerate into the spinal cord through the dorsal root entry zone (Carlstedt, 1988). Recently, this 3-day "critical period" was correlated to the distribution of CS-PG and CT (Pindzola et al., 1993). At late embryonic and early postnatal stages, both CT and CS-PG were colocalized to a small group of astroglia in the midline of the spinal cord, just lateral to the roof plate. Between postnatal days 1 and 3, the expression of these molecules spreads laterally through the dorsal root entry zone. Thus, the temporal distribution of these inhibitory molecules correlates with the inability of axons to regenerate into the dorsal root entry zone after postnatal 3.

Effect of Glia-Associated Proteoglycans on Neurite Outgrowth

In order to determine whether the expression of these particular molecules in areas of gliosis was directly involved in inhibiting neurite outgrowth, filters were implanted at various ages and then removed from the cortex, placed in culture, and used as a substrate for dissociated chick retinal ganglion cells (McKeon et al., 1991; McKeon and Silver, 1992). Prior to seeding these neurons, filters were treated with protease-free chondroitinase ABC in order to digest the GAG side chains linked to the core protein. After 2 days, neurite length was measured and compared to the length of neurites on untreated filters. When neuritic lengths were compared between treated and untreated filters removed from 30-day-old animals implanted as neonates, no differences were found, consistent with the limited expression of proteoglycans in young animals. In contrast, a significant increase in the amount of neurite outgrowth occurred on treated versus untreated filters removed from the cerebral cortex of adult animals 30 days postimplantation. Interestingly, the removal of an inhibitory component of the glial scar by chondroitinase ABC digestion results in an increased amount of neurite outgrowth by unmasking growth-promoting ECM, that is, laminin. Specifically, when neurons seeded on chondroitinase-treated filters are grown in the presence of antibodies to the $\beta1,\beta2$ chains of laminin neurite outgrowth is again significantly reduced when compared to the amount of outgrowth that occurs on chondroitinase treated filters alone (McKeon et al., 1994). These experiments demonstrate that the expression of certain CS-PGs can result in a dramatic decrease in neurite outgrowth on an astrocytic substrate by interacting with ECM molecules. The mechanism of this interaction is not yet clear, but may be due to the highly negative charge of the sulfated proteoglycan, stearic hinderance, or binding to an as yet unidentified receptor. Finally, these data are consistent with a recent report on the expression of both HS-PG and CS-PG following kainic acid-induced gliosis in the hippocampus (Bovolenta et al., 1993). When membranes isolated from injured versus uninjured hippocampi were used as a substrate for neurite outgrowth, significantly less outgrowth occurred on the injured membranes. Neurite outgrowth was improved following treatment of the inhibitory membranes with either heparinase or chondroitinase. The change in neurite outgrowth was greater following heparinase treatment, suggesting that HS-PG was the major inhibitory PG in this system.

WHAT IS THE STIMULUS FOR THE ONSET OF FUNCTIONALLY REACTIVE GLIOSIS?

Stimulation of Astrocyte-Associated Proteoglycan Deposition by β-Amyloid in an In Vitro *Model of Alzheimer's Disease and After Cortical Injury*

In addition to the intense glial response seen after CNS injury, reactive gliosis has also been suggested to be a normal consequence of aging (Duffy et al., 1980; Mancardi et al., 1983) and may play a role in the development of pathologic lesions in Alzheimer's disease (Frederickson, 1992). This hypothesis stems from the observation that there are increased numbers of intensely GFAP-positive glia associated with β-amyloid containing senile plaques as opposed to the number of glia around presenile plaques in the brains of adult humans and aged animals without dementia (Duffy et al., 1980; Nieto-Sampedro, 1987). In fact, it has been suggested that interactions between astrocytes and β-amyloid-containing plaques may be triggered by continued β-amyloid deposition (Mandybur and Chuirazzi, 1990). Finally, since various sulfated proteoglycans have been found to be associated with β-amyloid fibrils (Young et al., 1989; Snow et al., 1992), it has recently been suggested that reactive glia may be contributing to senile plaque formation through interactions via these extracellular matrix components (Canning et al., 1993).

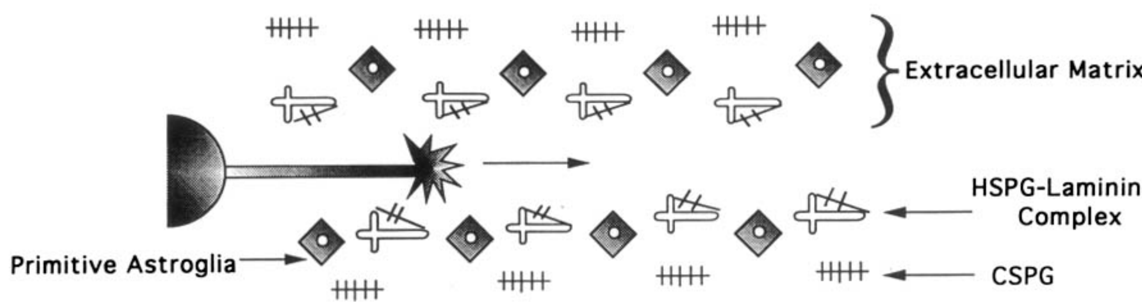
In order to test whether β-amyloid can induce functionally reactive (i.e., growth inhibitory) gliosis, Canning et al. (1993) cultured cortical neurons or astrocytes on synthetic peptides of the amino acid sequence of the β-amyloid molecule, which had been immobilized to a nitrocellulose substrate. Neurons adhered preferentially to peptides containing the 1-40 amino acid sequence rather than the surrounding laminin substrate. Furthermore, it was determined that this affinity was most likely due to the 25-35 amino acid sequence of the β-amyloid molecule.

In contrast to neuronal adhesion, cortical astrocytes, when placed in culture, initially did not show any preference for the various peptides used versus laminin. However, astrocytes on the surfaces of the 1-40 and 25-35 amino acid peptides were highly motile in relation to cells that had attached away from the β-amyloid peptide spot. The glial response was considered specific for the 1-40 sequence, since neither the 1-28 sequence nor scrambled peptides caused any reactive effects. Consistent with the hypothesis that astrocytes may interact with β-amyloid by depositing extracellular proteoglycans, Canning et al. (1993) next stained peptide spots that had been "conditioned" by astrocytes for up to 14 days. With time, there was an increasing amount of a CS-PG deposited both on and around the circumference of the peptide spot. Significantly, when cortical neurons were seeded onto the glial conditioned β-amyloid spots, they failed to show any adhesive preference, in contrast to their behavior on unconditioned spots, rather tending to adhere in higher numbers to the surrounding laminin. These data suggest that the surface of the peptide had now become an unfavorable substrate for neuronal attachment, due possibly by the deposition of proteoglycans by "reactive" astroglia.

The observation that exposure to specific amyloid peptides can induce the deposition of CS-PG *in vitro* is supported by recent *in vivo* evidence (Canning et al., 1993). When Millipore filters are implanted into the cortex of neonatal animals, there is little CS-PG staining even after 30 days postimplantation (McKeon et al., 1991). However, when β-amyloid-containing filters are implanted into neonates, there is a dramatic increase in the amount of CS-PG staining after 30 days, suggesting that glia within the cortex of young animals respond to the presence of amyloid by depositing increased amounts of CS-PG. This is consistent with a number of recent reports that have demonstrated a rapid appearance of β-amyloid precursor protein (APP) associated with reactive astrocytes after cortical injury in adult animals (Siman et al., 1989; Kawarabayashi et al., 1991; Otsuka et al., 1991), where there is also increased deposition of CS-PG (McKeon et al., 1991). Whether neonatal animals respond to trauma by depositing β-APP is not known. Finally, the above data, along with the finding that CS-PG is associated with astrocytes localized at the periphery of senile plaque cores in the brains of human patients with confirmed Alzheimer's disease (Canning et al., 1993), lend support to the hypothesis that β-amyloid may act as a general trigger for reactive gliosis in the CNS. This stimulus may result in the deposition of a variety of molecules inhibitory to axon outgrowth and survival and thus may play a role in the onset of this, and possibly other, neurodegenerative diseases.

TOWARD A THEORY OF NEURON/GLIAL CELL INTERACTIONS MEDIATED BY EXTRACELLULAR MATRIX MOLECULES

It is now evident that astroglia have many different functions within the CNS during development and in adult animals (Figure 27–1). These functions include expressing various cell or substrate adhesive



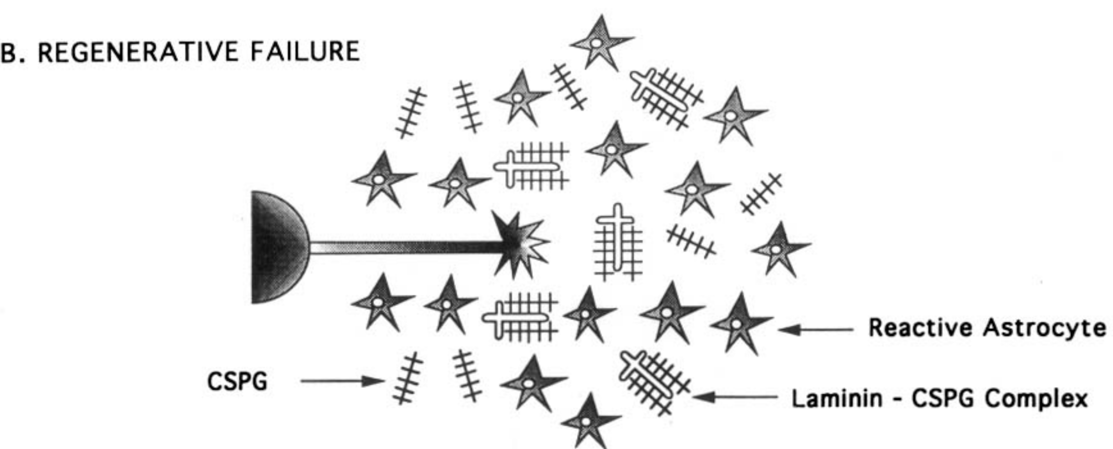


FIG. 27-1. Possible role of glial-derived extracellular matrix in neurite outgrowth during development and regenerative failure. **(A)** In certain regions during development, pioneering neurites may grow along preformed pathways of neuroepithelial cells, which later develop into astroglia. These primitive glia deposit laminin as part of the surrounding extracellular matrix (ECM). In addition to laminin, these cells express cell adhesion molecules (e.g., N-CAM and N-cadherin) on their surface. The combined expression of these growth-promoting molecules may help to direct growing neurites to specific regions of the developing brain. When complexed with heparan sulfate proteoglycan (HS-PG), the neurite growth-promoting capability of laminin is significantly increased. Alternatively, other proteoglycans, that is, chondroitin sulfate proteoglycan (CS-PG) and keratan sulfate proteoglycan, may form barriers to growing neurites in areas of the ECM surrounding these pathways, thus helping to direct neurite outgrowth by restricting the areas where neurites can grow. **(B)** During disease or after trauma to the CNS, astrocytes react by proliferating in a disorderly fashion around the area of damage. The density of reactive astrocytes has been suggested to form a physical barrier to regenerating axons. Also associated with the astrocytic response to injury is the increased deposition of CS-PG within the ECM. The increased expression of this putative growth inhibitory proteoglycan has been implicated as one reason for the failure of damaged axons to regenerate. However, the expression of growth promoting ECM molecules, specifically laminin, is also increased on the surface of the reactive glia, as well as part of the reestablished basal lamina. In this model, CS-PG may complex with laminin and limit its accessability to regenerating axons, thereby forming a molecular barrier to growing neurites. Removal of the CS glycosaminoglycan side chains from the native proteoglycan results in an increased amount of neurite outgrowth. This change in neurite outgrowth is reflective of an increased accessability to laminin, since it can be blocked by antilaminin antibodies. Thus, CS-PG may inhibit regenerating axons directly by serving as a poor substrate for neurite outgrowth or indirectly by complexing with trophic factors or growth promoting ECM molecules, limiting the accessability of these substances to regenerating axons.

molecules along pathways of developing axon tracts. Alternatively, certain populations of astrocytes can express other extracellular matrix molecules, whether during development, after injury, or during degenerative diseases, that are inhibitory for axon outgrowth. The best examined of these inhibitory molecules are the family of PGs, specifically chondroitin sulfate-containing PGs. However, the specific mechanism by which CS-PG inhibits neurite outgrowth is still unclear, although it is likely that they interact with ECM molecules and attenuate their growth-promoting properties.

Aside from inhibiting neurite outgrowth *in vitro* and being present in barrier areas *in vivo,* PGs are known to interact with ECM molecules. For example, heparan sulfate-proteoglycan (HS-PG) promotes neurite outgrowth by complexing with laminin (Dow et al., 1991), in contrast to the proposed in-

teractions between laminin and CS-PG. Thus, while the number and diversity of the proteoglycans in the CNS is only recently becoming clear (Herndon and Lander, 1990), it is likely that many of these PGs are involved in different functions during development and/or regenerative failure. One possibility is that the interaction between neurons and glia is mediated by the combined expression of each of these molecules, both growth-promoting and growth-inhibitory. While each of these molecules may affect axon outgrowth in a specific manner when used in isolation, because of the interaction between these different types of molecules, the final amount of neurite outgrowth that occurs after injury or during disease may be determined by the predominant type or amount of growth-promoting versus growth-inhibitory molecules ultimately expressed. Thus, for example, CS-PG may inhibit neurite outgrowth after injury directly by influencing the ability of axons to grow, but also indirectly by complexing with both growth-promoting ECM and trophic molecules, making them less available to growing neurites. Whatever the mechanism, increasing our knowledge of the specific surface and extracellular matrix molecules associated with astroglia will enhance our understanding of the role these cells and molecules play during development and pathological conditions.

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28 Tenascin and janusin: glial recognition molecules involved in neural development and regeneration

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NEURAL EXTRACELLULAR MATRIX GLYCOPROTEINS CONTRIBUTE TO THE REGULATION OF NEUROHISTOGENESIS

The development of the central nervous system (CNS) of vertebrates unfolds as a finely tuned sequence of specifiable steps. Several key events have been distinguished, namely, proliferation of epithelial stem cells, emigration of neuronal precursors from the ventricular zone to their final destination, formation of neuronal aggregates—the future nuclei—and of neuronal layers, establishment of neuronal projections to targets of innervation, neuronal cell death, and, finally, pruning and rearrangement of processes. These developmental events are subject to a tight network of regulatory mechanisms involving specific intercellular, membrane-mediated interactions, the presence of limiting amounts of growth factors and electrical activity of the nervous tissue (for review see Jacobson, 1991). It is well established that the interplay of neurons with glia plays a pivotal role in this context. For example, there is evidence that glial cells are critically involved in the guidance of migrating neuronal precursors and of extending neuronal fiber projections (see Chapter 49) and in the segregation of forming neuronal aggregates and nuclei (see Chapter 27 and below). Cell adhesion molecules and components of the extracellular matrix (ECM) mediate, at least in part, neuron-glia interactions. The majority of cell adhesion molecules hitherto described in the nervous system can be assigned to growing gene families, for example, the cadherin superfamily for the calcium-dependent and the immunoglobulin superfamily for the calcium-independent adhesion mechanism (Jessel, 1988; Rathjen and Jessell, 1991; Schachner, 1991; Takeichi, 1991). Furthermore, ECM components like laminin, fibronectin, or proteoglycans and integrin-type receptors are expressed in developing neural tissues (Sanes, 1989; Reichardt and Tomaselli, 1991). There is increasing awareness that, in addition to conducive, adhesive interactions, antiadhesive, inhibitory, and/or repulsive influences are of importance for the generation of ordered neural structures (Patterson, 1988; Keynes and Cook, 1992). Indeed, membrane-bound inhibitors have been described and are implicated in development and in degeneration and regeneration phenomena (see Chapters 27, 57, and 60). Also, a growing family of extracellular matrix glycoproteins, originally called J1 (Kruse et al., 1985), has been discovered in developing neural tissues which are of particular interest because they seem to embody both inhibitory and stimulatory properties for neuronal differentiation. Systematic study of these molecules might therefore deepen our understanding of cellular mechanisms underlying both growth and avoidance behaviors.

J1 COMPRISES TENASCIN AND JANUSIN

The J1 family of molecules was discovered as a constituent of the "rest-L2" fraction of L2/HNK-1-positive glycoproteins purified from detergent extracts of adult mouse CNS tissues. Polyclonal antibodies to a prominent 160 kD component of "rest-L2" react in Western blot experiments with four major components of 160 kD, 180 kD, 200 kD, and 220 kD apparent relative molecular mass (M_r), which show differential expression during CNS development. J1-200/220 is expressed during embryonal and early postnatal stages of development, while J1-160/180 reaches higher expression levels after birth and persists into adulthood. Immunoprecipitation studies demonstrated that J1-200/220 is produced by astrocytes *in vitro* (Kruse et al., 1985; Faissner et al., 1988). Polyclonal J1-antibodies interfere with the binding of granule cell neurons to astrocyte surfaces in a short-term *in vitro* assay (Keilhauer et al., 1985), suggesting that J1-200/220 mediates neuron-astrocyte interactions (Kruse et al., 1985). Subsequent systematic immunochemical

studies led to the conclusion that the J1-molecules consist of two independent entities, the pairs of glycoproteins J1-160/180 and J1-200/220, which contain small, but immunologically detectable homologies. J1-200/220 is the mouse homologue of tenascin, a growing family of extracellular matrix molecules (Faissner et al., 1988). J1-160/180 has been renamed janusin and constitutes an independent class of extracellular matrix glycoproteins (Pesheva et al., 1989, 1993; Fuss et al., 1993). The putative homologue of mammalian janusin in the chicken has been termed *restrictin* (Rathjen et al., 1991; Nörenberg et al., 1992). Increasing evidence suggests that both janusin and tenascin play a crucial role in mediating glial influences on neuronal and glial cell behavior.

TENASCIN GLYCOPROTEINS REPRESENT A DISTINCT GENE FAMILY OF EXTRACELLULAR MATRIX COMPONENTS

Tenascin glycoproteins of the ECM (Chiquet-Ehrismann et al., 1986) have been independently discovered by several groups and named myotendinous antigen (Chiquet and Fambrough, 1984a, 1984b), human glioma-mesenchymal extracellular matrix (GMEM) antigen (Bourdon et al., 1983, 1985), hexabrachion (Erickson and Inglesias, 1984; Erickson and Taylor, 1987), cytotactin (Grumet et al., 1985), J1-200/220 (Kruse et al., 1985, Faissner et al., 1988), J1/tenascin (Faissner and Kruse, 1990), and polypeptide 150/225 (Gulcher et al., 1986), and will be referred to as tenascin in this chapter. Tenascin is transiently expressed in developing organs such as the CNS (Kruse et al., 1985, Grumet et al., 1985, Gulcher et al., 1986), the kidney (Aufderheide et al., 1987), the intestine (Aufderheide and Ekblom, 1988), and the mammary gland (Chiquet-Ehrismann et al., 1986), to name just a few (reviewed in Erickson and Bourdon, 1989). In most of these cases, tenascin is confined to circumscribed areas in a "site-restricted" fashion, for example, to areas of epithelial-mesenchymal induction (Crossin et al., 1986). Primary derived amino acid sequences have been reported for chicken (Jones et al., 1989; Spring et al., 1989), mouse (Saga et al., 1991; Weller et al., 1991), and human tenascin (Gulcher et al., 1989, Siri et al., 1991). The glycoproteins comprise a cysteine-rich segment at the amino terminus, subsequent 13½ epithelial growth factor (EGF)-type repeats in the chicken and 14½ EGF-type repeats in mouse and human, followed by eight fibronectin type III (FNIII) homologous repeats and homologies to fibrinogen β and γ at the carboxyl terminus. Several isoforms have been described, which contain alternatively spliced FNIII repeats between the fifth and the sixth FNIII repeat of the basic structure. The interspecies homology between additionally inserted analogous FNIII repeats reaches 90%, while the intraspecies homology between FNIII modules is around 40%. Theoretically, a wealth of tenascin isoforms could potentially be encoded by various combinations of alternatively spliced FNIII repeats, but only a restricted number has hitherto been described in the mouse (Figure 28-1). Biochemically, tenascin glycoproteins display apparent molecular weights ranging from 190 kD to 320 kD and are linked to multimers by disulfide bridges at the amino terminal end (Chiquet-Ehrismann et al., 1986; Aufderheide et al., 1987; Taylor et al., 1989; Faissner and Kruse, 1990). The prevalent configuration of native tenascin is a hexamer, which appears as six-armed structure, a so-called hexabrachion, upon rotary shadowing and electron microscopy (Erickson and Inglesias, 1984, Vaughan et al., 1987). The apparent molecular mass of the hexabrachion is about 1000 kD, and it sediments with 6 S in glycerol gradients (Taylor et al., 1989). Tenascin glycoproteins from CNS tissues express the L2/HNK-1 carbohydrate epitope, in contrast to the mesenchymally derived glycoprotein (Kruse et al., 1984, 1985; Chou et al., 1986; Probstmeier et al., 1990).

TENASCIN IS A COMPONENT OF TRANSIENT CENTRAL NERVOUS SYSTEM BOUNDARIES

In the developing chicken, tenascin is first visible at the gastrula stage. Subsequently, it appears in the basement membrane of the developing neural tube and notochord and its expression unfolds as cephalocaudal gradient in a defined temporal sequence (Crossin et al., 1986). At later stages tenascin is detectable in distinct areas of the developing CNS where its expression generally correlates with key events of neurohistogenesis like neuronal migration, segregation of neuronal assemblies, and extension of neuronal processes (Crossin et al., 1986; S. Bartsch et al., 1992; U. Bartsch et al., 1992). Although tenascin-immunoreactivity has also been detected on the surface of neurites most neuronal cell types do not express the glycoprotein by themselves. *In situ* hybridization studies have clearly established astrocytes as the preeminent source of tenascin in the developing brain (Prieto et al., 1990; S. Bartsch et al., 1992; Tucker, 1991; 1993; Tucker et al., 1993). In addition, tenascin transcripts have been reported in the ependymal layer of postnatal mice (Tucker et al., 1993) and in a minority of avian retina neurons

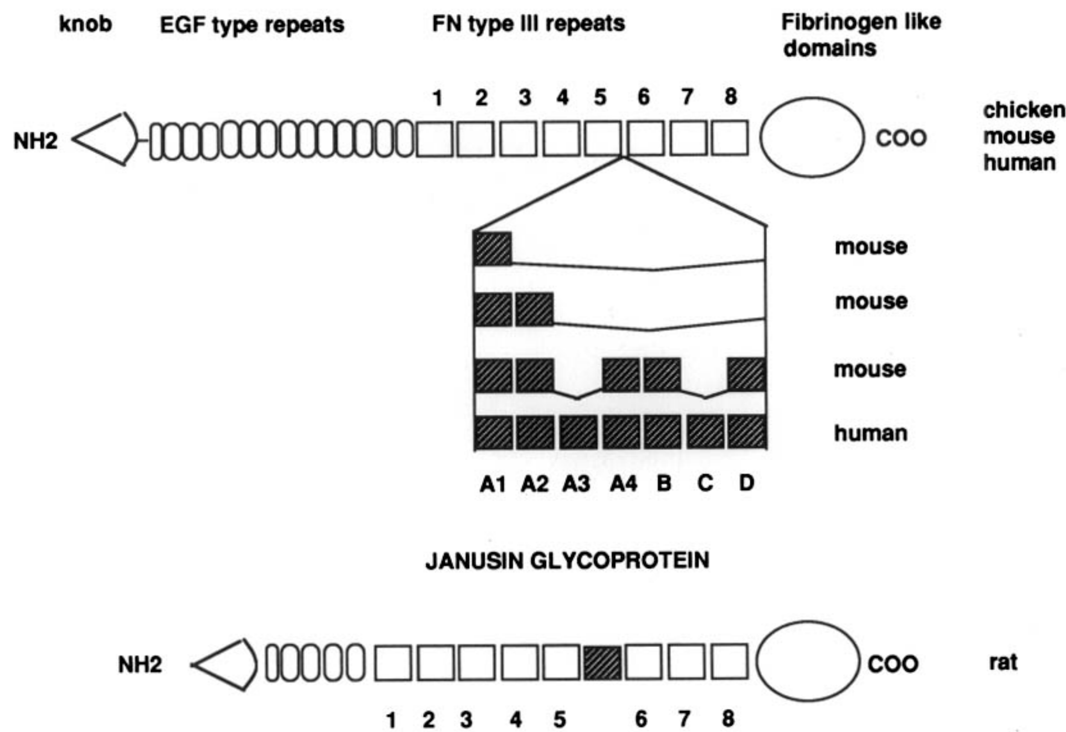


FIG. 28-1. Isoforms of tenascin. A summary of the known isoforms of mouse tenascin. The basic variant of the glycoprotein consists of a cysteine-rich amino terminus, a series of EGF-type repeats, 8 fibronectin type III (FNIII) homologous repeats (numbered 1 through 8) and homologies to fibrinogen β and γ at the carboxyl terminus in chicken, mouse, and human. The isoforms of mouse tenascin are generated by alternative splicing of additional repeats between FNIII module 5 and 6 of the smallest form. These are positioned according to the highest degree of homology with analogous motives of human tenascin, where 7 additional FNIII repeats have been discovered in the largest known isoform (designated *A1–A4*, *B*, *C*, and *D* following the suggestion of Aukhil et al., 1993). The scheme is based on sequence data published in the literature (Jones et al., 1989; Spring et al., 1989; Gulcher et al., 1989; Saga et al., 1991; Weller et al., 1991). Theoretically, a large number of variants is conceivable. For example, 7 distinct isoforms could be created with one additional FNIII repeat. It is currently unknown whether this potential is realized *in vivo*. The M_r's of the glycoproteins range from 190 for the smallest variant to 320 for the largest isoform described in humans (Erickson and Bourdon, 1989). For further details see text.

(Tucker, 1991). Analysis of the differential distribution of transcripts for tenascin isoforms as compared to the basic, nonalternatively spliced variant has revealed a correlation of the expression of the high M_r forms with events of cell proliferation, cell migration, and process outgrowth (Tucker, 1993). It had been noted that prospective functional neurophysiological processing units are transiently demarcated by glial cells during cortical development. These glial boundaries could be visualized with the lectin PNA whose binding site displays a locally restricted expression, for example, in the somatosensory barrel field (Steindler and Cooper, 1987; Cooper and Steindler, 1986a, 1986b). Recently, the discovery that tenascin immunoreactivity colocalizes with the lectin in several cases and transiently delineates barrels during the period of thalamocortical afferent ingrowth has considerably advanced our knowledge about the molecular composition of boundaries in neural tissues (Crossin et al., 1989, 1990; Steindler et al., 1989a, 1989b, 1990; Steindler, 1993) (Figure 28-2). Tenascin glycoproteins from postnatal mouse brain, however, do not display a PNA-binding site. In fact, the glial boundaries in the somatosensory cortex contain additional molecular components which colocalize with tenascin in these areas, such as the PNA-binding chondroitin sulfate proteoglycan CTBP (Crossin et al., 1989). Discrete distribution of tenascin glycoproteins has also been detected in other areas of the developing nervous

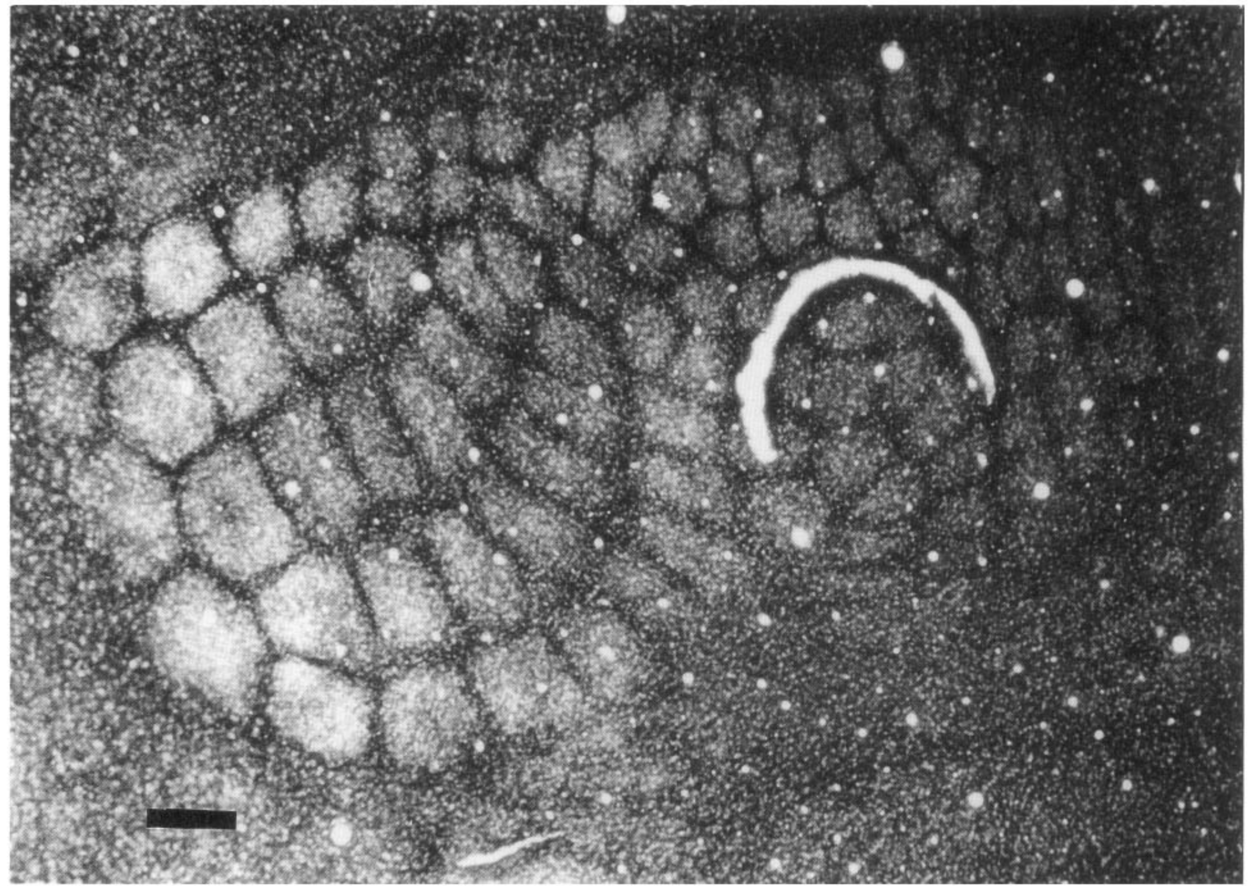


FIG. 28-2. Expression of tenascin glycoproteins in the somatosensory cortex. Top view of a flattened tangential section from postnatal day 6 somatosensory cortex of the mouse. Tenascin distribution was revealed by polyclonal antitenascin and peroxidase-derivatized anti-rabbit antibodies. Note that tenascin depositions delineate the boundaries of future barrels in the somatosensory barrel field. Bar: 200 μm. [This figure was generously provided by Dr. D. A. Steindler.]

system. For example, tenascin depositions follow the subdivision between patch and matrix compartments of the forming striosome or of fiber tracts in the internal capsule and separate functional compartments in nuclei of the brainstem, for example, the trigeminal nucleus (Steindler et al., 1990; Laywell and Steindler, 1991; O'Brien et al., 1992; Steindler, 1993), and tenascin boundaries have also been described in the forming peripheral nerve (Martini and Schachner, 1991). In all of these cases, the expression of tenascin is transient, and the glycoproteins are virtually undetectable in most of the adult, mature mouse CNS and PNS structures. In contrast, tenascin could still be detected in several adult CNS regions such as cerebellum, olfactory bulb, retina, and hippocampus (Miragall et al., 1990; S. Bartsch et al., 1992; U. Bartsch et al., 1992, 1993; U. Dörries and M. Schachner, unpublished observations). In addition, a persisting boundary of tenascin-immunoreactivity has, for instance, been revealed in the optic nerve head (U. Bartsch et al., 1994). Interestingly, this structure is believed to prevent immigration of oligodendrocyte precursors into the nonmyelinated retina (Small et al., 1987). It should be mentioned here that tenascin expression, however, is not discrete in all parts of the CNS at any stage of neural development. For example, rather uniform distribution of tenascin has been reported in places and at times of extensive axon outgrowth in mouse cerebellum (S. Bartsch et al., 1992), in mouse optic tectum (S. Bartsch, U. Bartsch, and M. Schachner, unpublished observations), in mouse cortex prior to and during thalamocortical afferent ingrowth (Godfraind et al., 1988; Jhaveri et al., 1991; Sheppard et al., 1991), and in the adult regenerating mouse sciatic nerve (Daniloff et al., 1989, Martini et al., 1990). Thus, both neuronal cell bodies and axonal growth cones seem able to cruise through homogeneous tenascin-containing territories *in vivo*.

TENASCIN EXPRESSION IS REGULATED BY VARIOUS FACTORS

The factors that regulate the timing and the topography of tenascin expression are only partially known. The tenascin gene promoter contains a functional binding site for the homeobox transcription factor Evx-1 and an AP-1/(TRE) site, which may be activated by cellular signal transduction events (Jones et al., 1990, 1992). Several growth factors have been reported to stimulate tenascin expression, for example, TGF-β1, NGF, bFGF, and angiotensin II, which enhance tenascin expression in embryonic fibroblasts, C6 glioma, Swiss 3T3 cells (a fibroblastoid cell line), and vascular smooth muscle cells, respectively (Pearson et al., 1988; Yavin et al., 1991; Sharifi et al., 1992; Tucker et al., 1993). In the case of 3T3 cells, bFGF preferentially induces the high M_r isoforms on both the protein and the message levels, while TGF-β increases the expression of the 190 kD basic variant (Tucker et al., 1993). In the somatosensory barrel field, the appearance of tenascin boundaries is concomitant or consecutive to the ingrowth of thalamocortical projections. The hollowing out of tenascin immunoreactivity in the barrel field might indicate that the tenascin boundaries in this area are sculptured by afferent fiber systems (Jhaveri et al., 1991; Steindler et al., 1989b). The dependence of boundary formation on neuronal afferents in striosomes has been experimentally demonstrated by selective lesioning of the nigrostriatal pathway (O'Brien et al., 1992). Consistent with these observations recent data show that fibers growing out from retinal explants induce downregulation of tenascin in astrocyte monolayers *in vitro* (Ard et al., 1993). The neuronal factor(s) presumably responsible for the establishment of tenascin patterns and the role of neuronal activity are as yet unknown. Finally, interspecies comparison has shown that the regulation of tenascin expression displays a certain degree of malleability during phylogeny. For example, human adult metencephalon does not show tenascin immunoreactivity, while bovine adult metencephalon displays substantial levels of the molecule (Rettig et al., 1992).

JANUSIN GLYCOPROTEINS BELONG TO THE SAME GENE FAMILY AS TENASCIN

Janusin, originally the J1-160/180 constituent of the J1 family of molecules (which is comprising tenascin and janusin glycoproteins), is a further member of the tenascin gene family (Erickson, 1993b) expressed in neural tissues and consists of two major glycoproteins of 160 kD and 180 kD apparent M_r (Kruse et al., 1985; Faissner et al., 1988; Pesheva et al., 1989; Morganti et al., 1990). Janusin immunochemically shares some epitopes with tenascin but represents a clearly distinct entity (Faissner et al., 1988; Pesheva et al., 1989), as underlined by the analysis of janusin cDNA sequences. Janusin consists of a cysteine-rich amino-terminal region followed by 4½ EGF-type repeats and nine FNIII repeats. The sequence is completed by homologies to fibrinogen β and γ. It is presently not known whether the isoforms of 160 kD and 180 kD are generated by alternative splicing of FNIII repeats (Fuss et al., 1993). The homology to tenascin FNIII repeats is about 70% at corresponding positions, underscoring the relatedness of both glycoproteins (Fuss et al., 1991, 1993) (Figure 28-1). In purified form both janusin and restrictin express the L2/HNK-1 epitope and form multimers under non-reducing conditions (Pesheva et al., 1989; Nörenberg et al., 1992). In particular, trimers and some mono-, di-, and tetramers have been observed for restrictin while the 160 kD and 180 kD forms of janusin form mainly mono- or dimers and trimers, respectively (Pesheva et al., 1989; Nörenberg et al., 1992). In the appropriate ionic milieu janusin assembles to large aggregates of interacting units which may be important in the context of myelinogenesis (Pesheva et al., 1991). Consistent with this view, janusin is expressed by oligodendrocytes at the onset of myelination (Pesheva et al., 1989, Wintergerst et al., 1993), where it possibly is involved in the wrapping of myelin loops in a self-binding mechanism (Pesheva et al., 1991). In myelin of young and adult animals, janusin remains detectable at nodes of Ranvier (ffrench-Constant et al., 1986), where it appears to mark a "microbarrier," interrupting the longitudinal expansion of myelin sheeths at the sites where axons, myelin, and astrocytes meet. Interestingly, a recent study has revealed that astrocytes or astrocyte-conditioned culture supernatants, PDGF and bFGF enhance expression of janusin on more differentiated, O1- and O10-positive oligodendrocyte surfaces, while dorsal root ganglia or spinal cord neurons induce janusin downregulation from both mature oligodendrocytes and their progenitors (Jung et al., 1993). It is noteworthy that Schwann cells, the myelin forming cells of the peripheral nervous system, do not express janusin at any developmental stage. It has therefore been speculated that the presence of janusin in myelin of the CNS and its absence in the PNS may be related to the capacities of axons to regenerate after infliction of a lesion in adult mammals. Finally, janusin protein and mRNA have been demonstrated in subpopulations of neurons in the central nervous

system (Rathjen et al., 1991; Fuss et al., 1993). The functional implications of janusin expression by certain types of neurons is, however, presently unknown.

TENASCIN AND JANUSIN DISPLAY REPULSIVE PROPERTIES FOR CENTRAL NERVOUS SYSTEM NEURONS

The distribution of tenascin in boundary-like structures that separate anatomically defined, functional processing units of the nervous system could indicate that it serves to segregate assembling neuronal modules from each other in these areas. Several *in vitro* studies support this hypothesis. Notably, tenascin glycoproteins *per se* are a bad substrate for neural cell culture. In fact, embryonic day (E)10 chicken neurons or glial cells fail to attach to tenascin when plated under normal gravity conditions and E11 chicken sympathetic, E14 rat mesencephalic, E18 rat hippocampal or postnatal day 6 mouse cerebellar neurons and E14 mouse cerebral astrocytes do not form monolayer cultures when the cells are plated on tenascin substrata and maintained for 24 hours *in vitro* (Friedlander et al., 1988; Faissner and Kruse, 1990; Wehrle and Chiquet, 1990). Rather than providing a favorable environment for neural cells, tenascin displays repulsive properties. For example, tenascin mixed with polyornithine induces neuronal aggregate formation and neurite fasciculation under high-density plating conditions of several types of embryonic and postnatal CNS neurons (Faissner and Kruse, 1990). Tenascin also induces the fasciculation and reduces the branching of neurites leaving E3 chicken spinal cord explants *in vitro* (Wehrle and Chiquet, 1990). Likewise, postnatal day 6 cerebellar neurons form less dense and aggregating cultures on mixed janusin-laminin substrates as compared to laminin alone (Pesheva et al., 1989). The induction of cell aggregation and neurite fasciculation on homogeneous tenascin-containing substrates could indicate that the adhesion forces between cell bodies and/or neurites and the culture substrate are reduced. Consistent with this interpretation, it has been documented that tenascin reduces the surface covered by the cell body *in vitro* in several cases. This so-called "antispreading effect" is paralleled by a reduction of the number of focal adhesion points which are believed to constitute the attachment sites of cells to their growth surface (Burridge et al., 1988). Antispreading effects of tenascin have been reported for both neural and nonneural cells, such as rat or mouse E18 hippocampal neurons (Lochter et al., 1991), mouse E11 fibroblast and mouse L929 cells (Chiquet-Ehrismann et al., 1988), neural crest cells (Tan et al., 1987; Epperlein, 1988; Mackie et al., 1988), mesodermal cells from gastrula stage embryos (Riou et al., 1990) and human melanoma cells (Halfter et al., 1989). The antispreading properties of tenascin are also observed when tenascin is combined with fibronectin, laminin, and polycations and is clearly documented when the cells are grown in the presence of tenascin added as soluble component to the culture medium rather than as component to the culture substrate (Tan et al., 1987; Chiquet-Ehrismann et al., 1988; Mackie et al., 1988; Faissner and Kruse, 1990; Riou et al., 1990; Lochter et al., 1991). Recently, also laminin and merosin, extracellular matrix molecules that possess binding sites for neuronal cell bodies and growth cones (Sanes, 1989; Reichardt and Tomaselli, 1991), have been assigned repulsive properties for olfactory neurons (Calof and Lander, 1991). Repulsive properties of extracellular matrix molecules might be required to regulate the strength of cellular interactions with the pericellular environment. Release of binding forces could be helpful for cell detachment, movement, and motility, and this might be the prominent biological function of components like tenascin, janusin, osteonectin/sparc, and thrombospondin, which display antiadhesive qualities in several nonneural tissue systems (reviewed in Sage and Bornstein, 1991; Chiquet-Ehrismann, 1991).

These repulsive and antispreading properties of tenascin glycoproteins are presumably mediated by cell binding site(s) in the molecule and complementary receptors at the surface of responsive cells. Indeed, erythrocytes, postnatal day 6 mouse cerebellar and E9 chicken retina neurons, E10 chicken embryo fibroblasts, mouse L929 and U251MG human glioma cells attach to tenascin in *short-term* cell-binding assays (Grumet et al., 1985; Kruse et al., 1985; Chiquet-Ehrismann, 1986; Friedlander et al., 1988; Bourdon and Ruoslahti, 1989; Nörenberg et al., 1992). Similarly, oligodendrocytes were found to interact with janusin in short-term adhesion assays (Morganti et al., 1990). It has therefore been proposed that initial cell binding to tenascin glycoproteins is required to trigger the antispreading or repulsion event in responsive cells, implying a two-step model of cell-tenascin or -janusin interactions. Initial recognition events could lead to downstream processes which, among others, perhaps involve cytoskeletal rearrangements (Lotz et al., 1989; Morganti et al., 1990; Murphy-Ullrich et al., 1991; Crossin, 1991; Faissner, 1993). At least three cell binding sites have been assigned to different FNIII and the fibrinogen-like domains of tenascin with the help of cDNA-de-

rived fusion proteins and/or monoclonal antibodies. These domains can be distinguished from areas presumably responsible for the antispreading and/or repulsive properties (Spring et al., 1989; Bourdon and Ruoslahti, 1989; Murphy-Ullrich et al., 1991; Prieto et al., 1992; Aukhil et al., 1993; Joshi et al., 1993; Sriramarao et al., 1993) (Figure 28-3).

The repulsive properties of tenascin are even more pronounced when the cells are exposed to patterned substrates. On these patterned substrates regions containing tenascin combined with a component supportive for cell attachment and differentiation such as the polycation poly-DL-ornithine or the ECM glycoproteins laminin or fibronectin alternate with areas covered with these latter components only. This design permits to monitor whether cells in choice situations are attracted to or dispersed from tenascin on the surface of a culture dish or coverslip. Under these conditions, embryonic and postnatal CNS neurons preferentially grow on tenascin-free parts of patterned tenascin/polyornithine coverslips (Faissner and Kruse, 1990). Likewise, neurites growing out from embryonic chicken dorsal root ganglion (DRG) explants avoid tenascin- or janusin-containing areas of patterned substrates constructed with laminin or fibronectin (Crossin et al., 1990; Taylor et al., 1993). The repulsive properties of tenascin *in vitro* are consistent with the possibility that these glycoproteins might separate neuronal assemblies and emerging fiber tracts *in situ*. Repulsive effects of tenascin have also been observed in neuron-glia cocultures where so-called "rocky" astrocytes produce circumscribed, locally enriched patches of tenascin, which deflect neurons and neurites (Grierson et al., 1990).

TENASCIN AND JANUSIN IN THE CONTROL OF NEURITOGENESIS

Despite their repulsive and/or antiadhesive effects on various cell types including neurons and their processes, also supportive roles of tenascin and/or janusin for histogenetic events have been documented. Thus, tenascin glycoproteins are thought to promote migration of mesodermal cells in amphibian gastrulation, of dispersing neural crest cells and their derivatives, for example, prospective corneal epithelia, and of granule cell neurons translocating from the external to the internal granule cell layer during cerebellar development (Chuong et al., 1987; Tan et al., 1987; Mackie et al., 1988; Bronner-Fraser, 1988; Riou et al., 1990; Kaplony et al., 1991; Tucker and McKay, 1991; S. Bartsch et al., 1992; Husmann et al., 1992). In the latter case a region presumably implied in granule cell migration could be allocated to

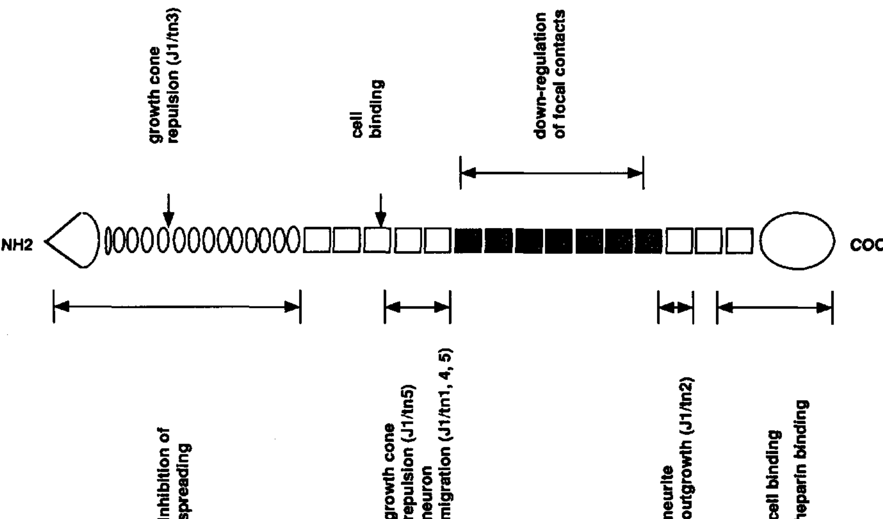
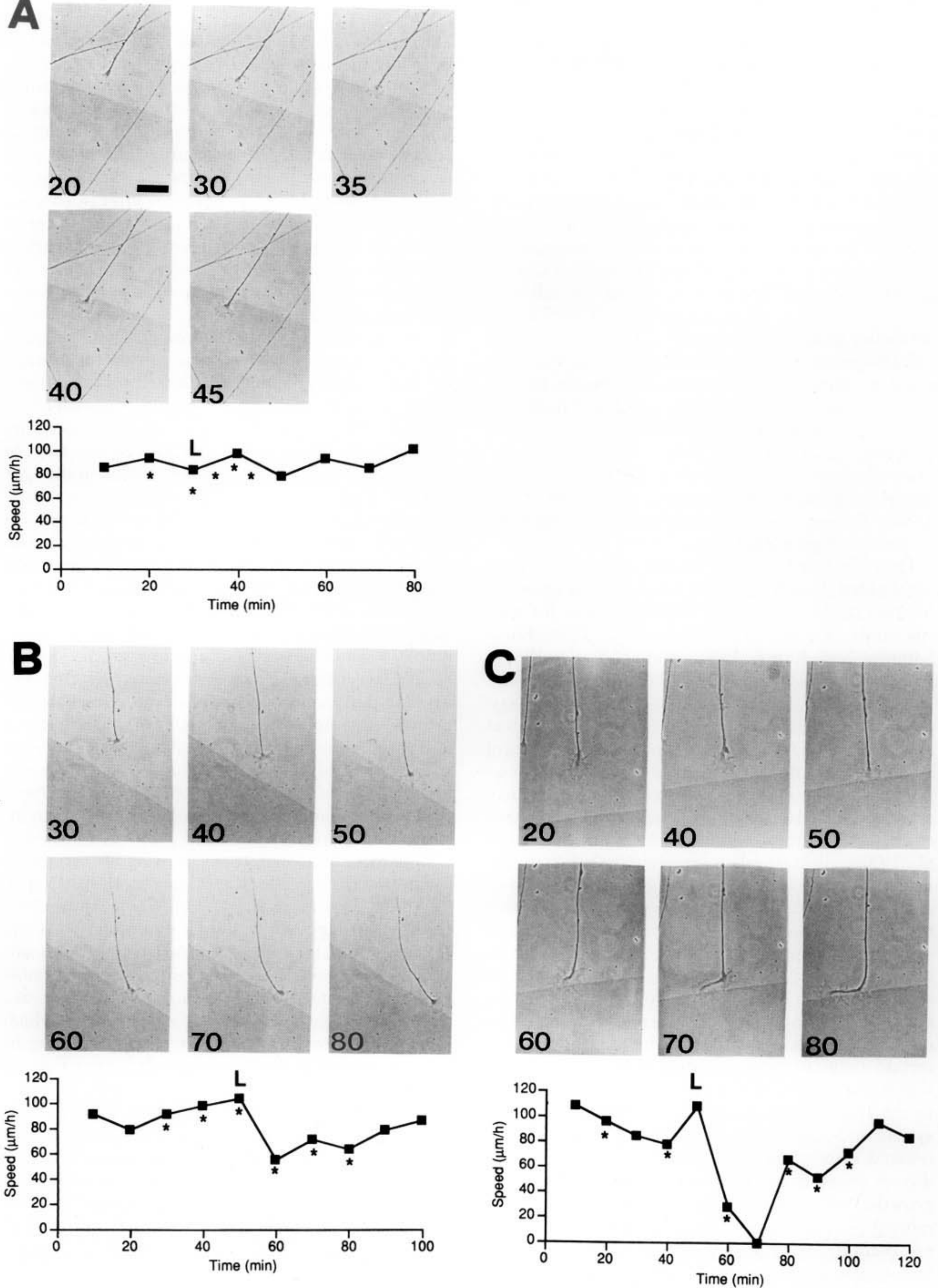


FIG. 28-3. Localization of functional domains of tenascin. Condensed results of several studies on structure-function interrelationships of tenascin glycoproteins. The functional domains are tentatively projected onto the largest known isoform. The map is based on the application of monoclonal antibodies with known binding sites, of fusion proteins produced in bacterial expression systems, and of proteolytic fragments of the glycoprotein in functional *in vitro* assays. The binding sites of the monoclonal antibodies J1/tn1-5 to mouse tenascin, which have been used in various perturbation assays, are indicated in parenthesis, next to the assignment of functional properties to the corresponding domains. For the meaning of the geometrical symbols see Figure 28-1, and for further details see text.

FNIII repeats 3 to 5 proximal of the alternative splice site by use of a panel of monoclonal antibodies in perturbation assays (Husmann et al., 1992) (Figure 28-3). Interestingly, dual, opposite effects of tenascin could also be demonstrated in these systems. Indeed, tenascin-containing substrata inhibit the emigration of satellite cells from E4 to E9 chicken DRG explants (Wehrle-Haller and Chiquet, 1993) and tenascin added as soluble component to the culture medium reduces neurite extension (Crossin et al., 1990, Lochter et al., 1991) and migration rates of neuroblastoma cells (Halfter et al., 1989) *in vitro* on a variety of substrates, for example, laminin and fibronectin. In view of these dual properties, the polarized expression of tenascin in the anterior as compared to the posterior somite during peripheral nervous system development may thus be part of a molecular scaffold involved in positioning neural crest derivatives (Mackie et al., 1988; Stern et al., 1989; Tan et al., 1987, 1991). In addition tenascin glycoproteins also possess neurite outgrowth promoting properties (Wehrle and Chiquet, 1990, Lochter et al., 1991, Husmann et al., 1992, Taylor et al., 1993, Lochter and Schachner, 1993, Wehrle-Haller and Chiquet, 1993). The promoting effect may depend on the developmental stage of the neurons, as shown for chicken DRG where tenascin-containing culture substrata stimulate neurite outgrowth from E4 to E6 and reduce or even inhibit fiber growth from E9 to E10 explants (Crossin et al., 1990; Taylor et al., 1993; Wehrle-Haller and Chiquet, 1993). Neurite outgrowth promotion by CNS neurons involves a region of the FNIII repeats around or downstream of the distal splice site and distinct from domains participating in cell-binding and repulsion (Lochter et al., 1991, Husmann et al., 1992) (Figure 28-3). The roles of tenascin and janusin in both the promotion and the guidance of neurite growth have been investigated in more detail in view of the fundamental importance of this process for the establishment of neuronal connections.

Careful analysis with time-lapse video microscopy has shown that growth cones of DRG and retinal ganglion neurons extending on laminin avoid growing into territories containing janusin and tenascin when confronted with sharp substrate boundaries of these glycoproteins. Thus both janusin and tenascin are repulsive for both peripheral (DRG neuron) and central (retinal ganglion neuron) growth cones which are given the choice between growing on these substrates or not (Figure 28-4). When DRG neuron growth cones are confronted with either janusin or tenascin in no-choice assays in the presence of laminin, they advance more on these substrates at a slightly faster rate than on laminin combined with a control substrate such as BSA. In contrast, retinal ganglion neuron growth cones are unable to advance on janusin- and tenascin-containing substrates in similar assays, which suggests that the ability of janusin and tenascin to promote neurite outgrowth might be cell type specific (Perez and Halfter, 1993; Taylor et al., 1993). Dorsal root and retinal ganglion cell growth cones are likely to encounter both tenascin and janusin during development (Crossin et al., 1986; Pesheva et al., 1989; Martini and Schachner, 1991; U. Bartsch et al., 1992; Wintergerst et al., 1993; U. Bartsch et al., 1993a, 1993b). That janusin is also repulsive for neurites in choice assays and is able to promote neurite outgrowth for at least one neuronal type, when offered as a uniform substrate, underscores the possibility that the structurally closely related glycoproteins janusin and tenascin may function to effect neurite outgrowth by similar mechanisms. The failure of monoclonal and polyclonal antibodies against janusin to neutralize the repulsive properties for advancing growth cones (Taylor et al., 1993) suggests that distinct epitopes on janusin are responsible for repulsion of neuronal cell bodies (Pesheva et al., 1989, Morganti et al., 1990), the initial recognition event between neurons and janusin (Morganti et al., 1990) and growth cone repulsion. Monoclonal tenascin antibodies J1/tn3 and J1/tn5 which bind to the EGF-like repeats and FNIII repeats 4 and 5, respectively, of mouse brain tenascin (Husmann et al., 1992, Figure 28-3), and polyclonal tenascin antibodies neutralize the repulsive properties of tenascin for advancing growth cones (Taylor et al., 1993). In contrast, monoclonal antibody J1/tn2 (Faissner and Kruse, 1990), which recognizes an epitope between FNIII repeats 6 and D (Lochter et

FIG. 28-4. Growth cone behavior at tenascin and janusin boundaries. Phase contrast images of representative retinal ganglion neuron growth cones approaching BSA (A), janusin (B), and tenascin (C) borders marked with colloidal gold-labeled BSA (*upper*) and corresponding velocity plots for these growth cones (*lower*). Numbers in the phase contrast images indicate time (in minutes) since the beginning of the film sequences, made between 24 and 36 hours after plating the retinal explants, and correspond to the times shown in the velocity plots, highlighted by *stars*. *L* denotes the times at which the growth cones first made lamellipodial contact with the border. Note that in Figure A the growth cone advances through the border with no change in velocity or morphology but that in Figures B and C, following contact with the border, the growth cones transiently reduce their rate of advance and turn to grow along the border with no apparent change in morphology. Scale bar in the first panel of Figure A represents 10 μm for all figures. [From Taylor et al. (1993), with permission. JNRS].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al., 1991, Figure 28-3) and monoclonal antibody J1/tn4, which recognizes an epitope within the 3rd and 4th FNIII repeats (Husmann et al., 1992) were ineffective. Antibody J1/tn2 blocks the neurite outgrowth-promoting properties of tenascin for three types of central nervous system neurons (Lochter et al., 1991, Husmann et al., 1992) but does not neutralize the repulsive properties of tenascin for cerebellar neuronal cell bodies (Faissner and Kruse, 1990). This suggests that different epitopes mediate the repulsion of DRG and retinal ganglion neuron growth cones and the repulsion of neuronal cell bodies. Monoclonal antibodies J1/tn4 and J1/tn5 retard cerebellar granule cell migration, in contrast to J1/tn3 (Husmann et al., 1992). Therefore at least two separate domains in the tenascin molecule participate in growth cone repulsion, which are both separate from the domain implied in neurite outgrowth promotion (Lochter et al., 1991). One of these domains, however, appears close to the site involved in cerebellar granule cell migration (Husmann et al., 1992). The location of these various domains is summarized in Figure 28-3.

Over the last few years several publications have highlighted the principle that neurite repulsion or inhibition might be an important mechanism for neurite guidance (e.g., Kapfhammer et al., 1986; Kapfhammer and Raper, 1987; Walter et al., 1987a, 1987b; Caroni and Schwab, 1988a, 1988b; Baier and Bonhoeffer, 1992; see also Patterson, 1988, Walter et al., 1990, Keynes and Cook, 1992, Schwab et al., 1993, for reviews) and janusin and tenascin could also function in this manner when present as boundaries. Janusin is expressed by oligodendrocytes and in central nervous system myelin (Pesheva et al., 1989; Wintergerst et al., 1993; U. Bartsch et al., 1993a), notoriously nonpermissive substrata for neurite outgrowth (Schwab and Caroni, 1988; Fawcett et al., 1989). Tenascin is found in the developing rodent central nervous system (see above) or in areas around stab wounds in the adult central nervous system (McKeon et al., 1991; Laywell et al., 1992; Masuda-Nakagawa et al., 1992). Janusin and tenascin might in addition function as promoters of neurite outgrowth, or at least allow neurite outgrowth, from certain types of neurons when they are expressed uniformly, such as appears to be the case of tenascin in the regenerating mammalian peripheral nervous system and certain regions in the developing central nervous system (see above). The apparently contradictory properties of janusin and tenascin in causing growth cone avoidance in choice assays, and yet promoting growth cone advance in no-choice assays, are reminiscent of a previous study of a repulsive factor present in the chick tectum (Walter et al., 1987a, 1987b). Axons from the chick temporal retina avoid membranes derived from the posterior tectum, an incorrect target tissue for these axons, when given the choice of growing on these membranes or those derived from the anterior tectum, their correct target tissue, but are able to grow as well on posterior as anterior tectal membranes when given no choice. A more recent publication indicates that a gradient of the repulsive factor present in posterior tectal cell membranes of at least 5% per 25 μm is required to prevent growth cone advance (Baier and Bonhoeffer, 1992). It would be of interest to determine whether the same is true for janusin and tenascin, although, to date, shallow gradients of these molecules have not been obvious *in vivo* (in contrast to sharp step gradients at tenascin boundaries). When DRG or retinal ganglion neuron growth cones contact sharp substrate boundaries of janusin and tenascin, the temporary reduction in the rate of growth cone advance is only very rarely, in the case of DRG growth cones, and never, in the case of retinal growth cones accompanied by complete growth cone collapse (Taylor et al., 1993). In the few cases where it was observed, complete growth cone collapse was transient and not reminiscent of the type of growth cone inhibition observed when growth cones contact other nonpermissive substrates, for example, oligodendrocytes (Bandtlow et al., 1990). Several recent studies have suggested that neurite avoidance of inappropriate substrates is mediated by growth cone collapse (Fawcett et al., 1989; Raper and Kapfhammer, 1990; Cox et al., 1990, Davies et al., 1990; Bandtlow et al., 1990; see also Walter et al., 1990; Keynes and Cook, 1992) and that growth cone collapse-inducing molecules can be demonstrated by incorporating them into liposomes and adding them to growth cones growing on permissive substrates (Raper and Kapfhammer, 1990, Cox et al., 1990, Davies et al., 1990). When janusin and tenascin are applied as soluble molecules to either DRG or retinal ganglion neuron growth cones advancing on laminin, no effect on growth cone morphology or rate of advance is seen (Taylor et al., 1993). It appears, therefore, that molecules which guide growth cones by repulsive or inhibitory mechanisms may produce different avoidance reactions in advancing growth cones involving either an interaction with the repellent molecule leading to a change in the direction of growth cone advance, as observed for janusin and tenascin or growth cone collapse, which may finally also result in redirection of neurite outgrowth. Similar mechanisms may be operating to repel DRG neuron growth cones from janusin and tenascin boundaries in choice experiments, and to speed growth cone advance in no-

choice experiments. The DRG neuron growth cone lamellipodia confronting janusin and tenascin borders in choice experiments undergo a localized collapse that can be compared to the collapsed morphology of rapidly advancing growth cones on uniform janusin- or tenascin-containing substrates. Thus, it could be envisaged that the localized lamellipodial collapse seen in DRG neuron growth cones at janusin and tenascin borders in choice experiments is due to a localized change in the concentration of a second messenger, which in turn mediates the localized lamellipodial collapse due to localized cytoskeletal rearrangement. Along these lines, the overall collapsed appearance of DRG neuron growth cones which find themselves surrounded by janusin- and tenascin-containing substrata, in no-choice experiments, could result from a global change in the concentration of the same second messenger and consequent global rearrangement of the growth cone cytoskeleton.

DIVERSE TENASCIN AND JANUSIN FUNCTIONS MAY REQUIRE VARIOUS RECEPTORS

Several receptors have been described which could contribute to mediate tenascin effects, for example integrins (Bourdon and Ruoslahti, 1989; Leahy et al., 1992; Sriramarao et al., 1993; Wehrle-Haller and Chiquet, 1993), the neural chondroitin sulfate proteoglycan CTBP (Hoffman and Edelman, 1987; Hoffman et al., 1988), the heparan sulfate proteoglycan syndecan (Salmivirta et al., 1991), sulfatide (Crossin and Edelman, 1992), and the Ig-superfamily member contactin/F3/F11 (Ranscht and Dours, 1988; Brümmendorf et al., 1989; Gennarini et al., 1989, 1991; Zisch et al., 1992). In addition, interactions of tenascin with other ECM components like fibronectin or collagens might modify the functional properties of the glycoprotein (Chiquet-Ehrismann et al., 1988; Marton et al., 1989; Lightner and Erickson, 1990; Faissner et al., 1990; Probstmeier et al., 1990; Chiquet et al., 1991). Tenascin glycoproteins hence appear as multimodular, multifunctional glycoproteins which exhibit distinct functional sites implied in cell-binding, in antispreading-repulsion events, in cell migration and in the control of neurite growth. In light of these results, tenascin glycoproteins could enclose forming neuronal assemblies, segregate outgrowing fiber tracts or aid in neuron migration during neural development. How the different domains of tenascin interact and which receptors are crucial in these processes remains at present elusive (discussed in Faissner, 1993; Faissner et al., 1994). Also, a proof that tenascin glycoproteins perform diverse functions *in situ* is still missing and will require direct perturbation in adequate *in vivo* systems. A recent experiment was not conclusive in this regard because inactivating the tenascin gene in the mouse does not overtly interfere with mouse development. Whether the fine structure of the nervous system is altered in the TN(−) mutants was, however, not addressed (Saga et al., 1992). Also, analysis has been confined to postnatal stages of development while the investigation of potential long-term effects of the knock-out is still pending. For these reasons, it seems premature to disregard tenascin as "junk protein" with superfluous and spurious expression (Erickson, 1993a).

In search for neuronal ligands mediating the repulsive response to janusin the F3/11 cell surface glycoprotein, a glycosylphosphatidyl-inositol anchored member of the immunoglobulin superfamily, has been identified (Gennarini et al., 1991; Pesheva et al., 1993). F3/11 mediates the initial recognition between a janusin substrate and cerebellar neurons or F3-transfected CHO cell. In cerebellar neurons, the interaction between F3/11 and janusin induces a repulsion consisting of the loss of substrate adhesion with time in culture and inhibition of neurite outgrowth. Antibody blocking experiments show that the avoidance response of neurites at janusin substrate borders is also mediated by F3/11. These results document for the first time a ligand-receptor pair involved in the repulsion of neurons. It is likely that particular mechanisms of signal transduction which are induced by F3/11 lead to the avoidance behavior of growth cones and neuronal cell bodies (Bixby and Harris, 1991). The linkage of an adhesion molecule of the Ig-superfamily to second-messenger systems has already been documented for L1 (Schuch et al., 1989, Atashi et al., 1992). It is interesting that F3/11 is also able to mediate adhesive responses, for example when transfected into CHO-cells (Gennarini et al., 1991), possibly involving another Ig-superfamily molecule, L1 (Kuhn et al., 1991). Consistent with these reports, F11/contactin, the chicken analogue of F3, has recently been shown to interact with restrictin and Ng-CAM, the chicken equivalents of janusin and L1, respectively (Brümmendorf et al., 1993). Future investigations will focus on the fine structure of functional tenascin- and janusin-domains, the identification of complementary receptors and the elucidation of second messenger pathways controlling the cellular responses to these glycoproteins.

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29 Growth factors and their receptors in the peripheral nervous system

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Our understanding of how growth factors and growth factor receptors are involved in the complex mechanisms regulating physiological development and maintenance of the peripheral nervous system has advanced considerably during the past few years. Several major steps in the field have been made: (1) the cloning of brain-derived neurotrophic factor (BDNF) (Leibrock et al., 1989), which led to the identification of the still growing gene family of neurotrophins (Barde, 1990), (2) the identification of the *trk* oncogene as the high-affinity receptor for nerve growth factor (NGF) (Kaplan et al., 1991a, 1991b; Klein et al., 1991a) and the subsequent identification of the *trk* tyrosine kinase gene family of receptor molecules for the neurotrophins (reviewed by Meakin and Shooter, 1992), (3) progress in elucidating how the expression of neurotrophic factors is physiologically regulated in the peripheral nervous system. The analysis of the role of IL-1 in the upregulation of NGF mRNA in peripheral nerves (Heumann et al., 1987b; Lindholm et al., 1987) after lesion was one of the first examples of how cytokines with a defined function in the immune system are involved in the regulation of neuronal regeneration mediated by neurotrophic factors such as NGF. Knowledge about the complex functions of growth factors in the peripheral nervous system is still rapidly increasing, in particular from experiments on transgenic animals with altered expression of these molecules.

This chapter focuses on the specific classes of neurotrophic factors (neurotrophins and ciliary neurotrophic factor), members of pluripotent fibroblast growth factor (FGF) and insulinlike growth factor (IGF) gene families, and the newly identified and molecularly characterized glial growth factors and their functions in the peripheral nervous system.

NEUROTROPHIC FACTORS

Neurotrophins

Neurotrophins are polypeptide molecules that regulate the development and maintenance of specific functions of different populations of nervous cells in the peripheral and central nervous systems (Barde, 1990). This still-growing family of molecules includes at the moment four different molecules:

1. The prototypic nerve growth factor (for details of its discovery see Chapter 30, this volume)
2. Brain-derived neurotrophic factor (Barde et al., 1982, Leibrock et al., 1989)
3. Neurotrophin-3 (NT-3) (Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990)
4. Neurotrophin-4/5 (NT-4/5) (Berkemeier et al., 1991; Hallböök et al., 1991)

In the case of NT-4, first identified and cloned from *Xenopus* (Hallböök et al., 1991), it was subsequently established that this molecule is the analogue of NT-5 (Ip et al., 1992), which had been cloned from human sources (Berkemeier et al., 1991) at about the same time as NT-4 was identified in *Xenopus*. These four factors share more than 30% sequence homology on the mature protein level, and they are relatively well conserved between mammals. Interestingly, the conserved regions of the four mature neurotrophins include the six cysteines, which are thought to play an essential role in the stabilization of the three-dimensional structure of these molecules. Indeed, when the three-dimensional structure for NGF was solved (McDonald and Hendrickson, 1993), it proved to be a long molecule consisting of three pairs of twisted antiparallel β-strands linked by four loop regions. The cysteine residues form an interlocked, knotlike motif at one end of the molecule, a structural feature since found to be shared by other proteins such as TGF-β2 and PDGF-BB (for review see McDonald et al., 1993). The variable domains between the different members of the gene family reside mainly in the four hairpin loops. They are thought to be responsible for determining the different receptor specificities of the four neurotrophins identified so far.

Specificity of Neurotrophins for Specific Neuronal Populations. *In vitro,* the neurotrophins show differ-

ential effects on populations of neurons whose axons project within peripheral nerves. Nerve growth factor, for example, supports the survival of cultured sympathetic neurons, whereas BDNF (Barde et al., 1982) and NT-4 (Berkemeier et al., 1991) are inactive. Recent evidence suggests that also NT-3 is more specific for *trk*C than for *trk*A or *trk*B (Ip et al., 1993b), thus confirming earlier studies showing that NT-3 might not physiologically support the survival of cultured sympathetic neurons (Hohn et al., 1990) and revising other reports (Glass et al., 1991; Soppet et al., 1991). The neuronal placode-derived sensory neurons of the embryonic day 8 chick nodose ganglion are only supported by BDNF (60% survival), NT-3 (30% survival) (Hohn et al., 1990), and NT-4 (Hallböök et al., 1991), but not by NGF. The actions of BDNF and NT-3 are additive (survival of more than 90% of the originally plated neurons from 8-day-old chick embryos), suggesting that they are mediated by different receptors.

In the case of the neural crest-derived sensory neurons of the dorsal root ganglion, there seem to be subpopulations of neurons that are responsive to either one or more of the neurotrophins. NGF supports about 50% of cultured embryonic day 8 chick dorsal root ganglionic neurons, BDNF supports about 45%, and NT-3 supports about 30% of the cultured neurons (Dechant et al., 1993). If two or all three factors are added to these cultures, additive survival effects are detectable, suggesting that these three neurotrophins act independently through different receptors on different populations of neurons within the embryonic day 8 chick dorsal root ganglia. These results suggest a high degree of specificity of the neurotrophins for responsive neurons, which is consistant with the regulatory roles of these factors on responsive neurons during development and in the adult (Barde, 1989, 1990).

The Receptors for Neurotrophins. Two receptors for NGF have been identified and characterized so far, namely, $p75^{NGFR}$ (reviewed by Meakin and Shooter, 1992) and the protooncogene *trk*A (Kaplan et al., 1991a, Kaplan et al., 1991b, Klein et al., 1991a). In addition, several members of the *trk* tyrosine kinase family have been shown to act as high-affinity receptors for the other neurotrophins, such as *trk*B (Middlemas et al., 1991) as a high-affinity receptor for BDNF (Klein et al., 1991b) and NT-4/5 (Klein et al., 1992), and *trk*C as a high-affinity receptor for NT-3 (Tsoulfas et al., 1993) (Figure 29-1). Original reports (Cordon-Cardo et al., 1991; Klein et al., 1991b; Squinto et al., 1991) have suggested that *trk*A and *trk*B might serve as signal transducing receptors with broader specificity for NGF and NT-3 or BDNF and NT-3, respectively. However, recent data suggest that both in neuronal cells and PC-12 cells, the specificity of *trk*B for BDNF and NT-4/5 seems to be much higher than for NT-3 (Ip et al., 1993b, Tsoulfas et al., 1993). Indeed, recent evidence from cultured embryonic chick nodose neurons has suggested that NT-3, which binds to *trk*B at high concentrations in the culture medium, competes with BDNF as a receptor antagonist rather than an agonist (Dechant et al., 1993). *In vivo*, similar observations have been made that are compatible with the assumption that NGF might act as a competitive antagonist on neurons expressing *trk*B and *trk*C. Survival rates of spinal and facial motoneurons in newborn rats after lesion are decreased rather than increased by NGF addition (Miyata et al., 1986; Sendtner et al., 1992a). Thus the biological responses of neurotrophins seem to be mediated by specific high-affinity receptors which involve the tyrosine kinase cell transmembrane proteins of the *trk* gene family.

It is still not very clear how the p75 neurotrophin receptor molecule contributes to the biological actions of neurotrophins (Meakin and Shooter, 1992). Protein 75, which has been shown to bind NGF, BDNF, and NT-3 with similar low affinity (Rodriguez-Tebar et al., 1990, 1992) does not have a tyrosine kinase domain within its cytoplasmic region. A consensus sequence for the binding of G proteins has been identified (Feinstein and Larhammer, 1990); however, the question is still open whether G proteins contribute to the biological actions of NGF in responsive neurons. Interestingly, the p75 low-affinity neurotrophin receptor is also expressed by glial cells within the peripheral nervous system (reviewed by Johnson et al., 1988). During development, Schwann cell precursor cells express high amounts of p75 protein. At later stages, when Schwann cells cease to divide and become myelinated, p75 expression is downregulated to undetectable levels. However, after lesion, this receptor molecule is again rapidly reexpressed by Schwann cells within the distal stump of the lesioned nerves. The physiological function of the low-affinity neurotrophin receptor on Schwann cells is not clear. Gene targeting (Lee et al., 1992) has shown that mice deficient in this protein are viable, and that they do not show any apparent defects of the sympathetic nervous system. Only sensory deficits are seen, particularly reduced heat sensitivity, which corresponds to decreased density of sensory nerve fibers in the skin of these mice. However, it is not yet clear whether the rate of neuronal loss is increased in the dorsal root ganglia or other neurotrophin-dependent neuronal cell population during the period of naturally

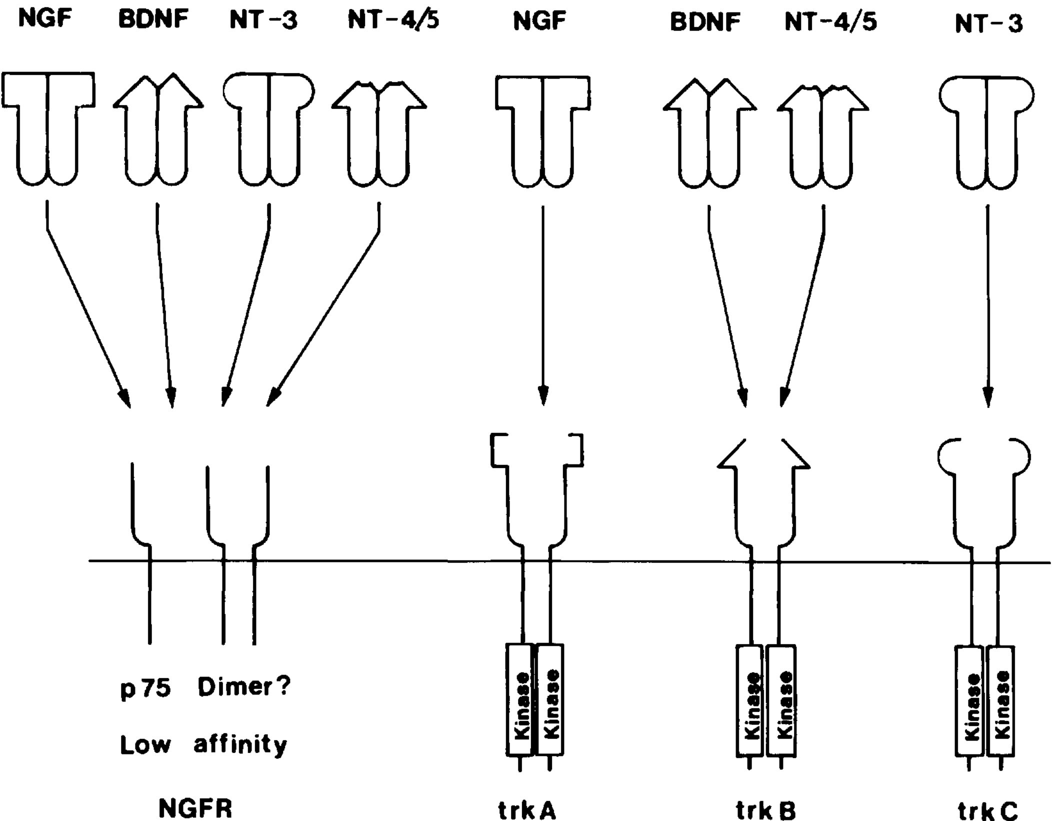

FIG. 29-1. Model of neurotrophin action on target cells. Neurotrophin homodimers activate specific *trk* tyrosin kinases by inducing receptor dimerization. All neurotrophins can bind with similar affinity to the low-affinity nerve growth factor receptor (p75). However, the biological function of this receptor is still unclear.

occurring cell death. The sensory deficits in this mouse could also be due to the lack of expression of p75 on Schwann cells during development, thus leading to indirect impairment of sensory fiber outgrowth to the skin.

In the case of the *trk* gene family of tyrosine kinases, it seems easier to match the temporal and spatial expression of these molecules with functional aspects. The expression of *trk*A on neurons correlates very well with NGF responsiveness (Carroll et al., 1992). Similarly, *trk*B and *trk*C tyrosine kinase expression seems to be restricted to neuronal cells known to be responsive to BDNF and NT-3 (Klein et al., 1989; 1990, Henderson et al., 1993), respectively. This is compatible with the assumption that the actions of neurotrophins on neuronal survival and function are primarily mediated by the *trk* tyrosine kinases.

Function of Target-Derived Neurotrophins in the Peripheral Nervous System. Nerve growth factor is the prototype of a target-derived neurotrophic molecule. Only low amounts of NGF mRNA are found in target tissues of responsive neurons, which, within the peripheral nervous system, are usually nonneuronal cells. These low amounts of NGF mRNA and protein in target tissues correspond to the low quantities of NGF protein detectable by highly sensitive immunoassays (reviewed by Barde, 1989). For example, in the adult rat iris, which is densely innervated by NGF-dependent sympathetic nerve fibers, about 1.9 ng of NGF per gram of tissue wet weight are found (Heumann et al., 1987a). During embryonic development, the low quantities of NGF protein do not seem to be sufficient to saturate the high-affinity binding sites of all dependent neurons, so that about half of them degenerate because of the limited availability of this factor (reviewed by Barde, 1989). NGF binds to dependent neurons via high-affinity receptors (K_d in the range of 10^{-11}M), which are taken up in membrane-bound vesicles. These vesicles, which still contain intact NGF protein, are retrogradely transported from the nerve terminal to the cell body. Signal transduction is initiated after NGF is bound to its high-affinity receptors (Meakin and Shooter, 1992). However, it is still unresolved whether signaling events are generated at the nerve terminals after binding of NGF to its receptor, or at later stages when the NGF-containing vesicles have been transported to the corresponding nerve cell

bodies. Many pieces of evidence have shown that NGF is important for the survival of neurons during a critical period of development when about half of the postmitotic neurons of the NGF-dependent sympathetic and neuronal crest-derived sensory neurons are eliminated (Barde, 1989, Oppenheim, 1985, Johnson et al., 1986). Furthermore, NGF from target tissues regulates functionally important features of the neurons during postnatal life, such as transmitter synthesis. In sympathetic neurons, levels of tyrosine hydroxylase are elevated after exogenous NGF addition, and the enzyme levels are downregulated after addition of neutralizing antibodies against this factor (reviewed by Thoenen and Barde, 1980). Similarly, in sensory neurons, substance P levels are dramatically reduced after addition of neutralizing antibodies against NGF (Otten et al., 1980), suggesting that NGF plays a key role in the regulation of transmitter synthesis by target tissues. Although methods for measurement of BDNF, NT-3, and NT-4 protein levels in peripheral tissues are not yet established, the levels of the corresponding mRNAs coding for these factors are in general consistent with a similar function of these factors for their responsive or dependent neurons, as established for NGF. For example, BDNF and NT-3 mRNAs are found in small but significant levels in gut, lung, and heart of adult mouse, tissues known to be strongly innervated by sensory neurons of the nodose ganglion (Hofer et al., 1990; Hohn et al., 1990; Maisonpierre et al., 1990). In addition, BDNF and NT-3 mRNAs are also expressed in skeletal muscle, the target of BDNF and NT-3 responsive motoneurons and proprioreceptive sensory neurons (Hohn et al., 1990; Maisonpierre et al., 1990; Koliatsos et al., 1993). The distribution of NT-4 mRNA expression in peripheral tissues is yet to be studied in detail. Preliminary studies have shown that very low amount of NT-4/5 mRNA are detectable in skeletal muscle (Berkemeier et al., 1991; Ip et al., 1992) and skin (Henderson et al., 1993, Ibanez et al., 1993), the targets of NT-4/5 responsive motor and sensory neurons. Relatively high levels of NT-4/5 mRNA are also found in ovary and thymus. However, the physiological role of NT-4/5 in these tissues remains to be established.

Expression of Neurotrophins by Neurons Within the Peripheral Nervous System: Potential Physiological Function. Recently it has been found that members of the neurotrophin gene family are also expressed by developing sensory and motor neurons. By *in situ* hybridization techniques, BDNF mRNA has been detected in developing dorsal root ganglionic sensory neurons from embryonic day 13 to adulthood (Ernfors and Persson, 1991). In addition, relatively high levels of NT-3 mRNA have been detected in spinal motoneurons of the rat between embryonic day 13 and birth. These results were unexpected, as both the sensory neurons and the spinal motoneurons have been shown to be responsive to these molecules. Indeed, on the basis of these results, the question has been raised whether these neuronally derived trophic factors could act locally in an autocrine or paracrine fashion. This is conceivable, as a significant population of the sensory neurons within the developing dorsal root ganglia are responsive to BDNF, both *in vitro* and *in vivo* (Hofer and Barde, 1988; Barde, 1990; Oppenheim et al., 1992b). Indeed, cultured sensory neurons from embryonic day 4.5 chick embryos, which are not yet dependent on neurotrophins for their survival but produce BDNF themselves, seem to depend on the endogenous BDNF for their maturation, as shown with BDNF antisense oligonucleotides in cell culture (Wright et al., 1992). These results argue in favor of the existence of autocrine actions of neurotrophins. However, it is still open as to whether such autocrine effects play a role during the period of physiologically occurring cell death.

As an alternative possibility, it has been suggested that the NT-3 produced in motoneurons would serve as a trophic factor for the proprioceptive sensory neurons innervating the ventral horn (Ernfors and Persson, 1991). This possibility is supported by the observation that transsection of the central processes of dorsal root sensory neurons in newborn rats results in significant rates of cell death in the affected dorsal root ganglia (Johnson et al., 1986), suggesting that the interruption of the central connection of sensory neurons leads to loss of trophic support from the central nervous system. However, it is not possible at the moment to draw final conclusions on the physiological consequences of the expression of NT-3 in motoneurons.

The Regulation of Nerve Growth Factor and Brain-Derived Neurotrophic Factor mRNA in Peripheral Nerves After Lesion. In nonneuronal cells of the adult peripheral nerve, neither NGF nor BDNF are expressed under physiological conditions (Heumann et al., 1987b, Meyer et al., 1992). However, after lesion of peripheral nerves, NGF mRNA is rapidly upregulated at the lesion site and in the distal stump (Figure 29-2). The increase of NGF mRNA in these two regions is biphasic: After an initial rapid increase during the first hours after lesion, NGF mRNA levels decrease again before a second, longer lasting, increase of NGF mRNA occurs. This second phase of NGF induction is due to interleukin-1 (IL-1) released

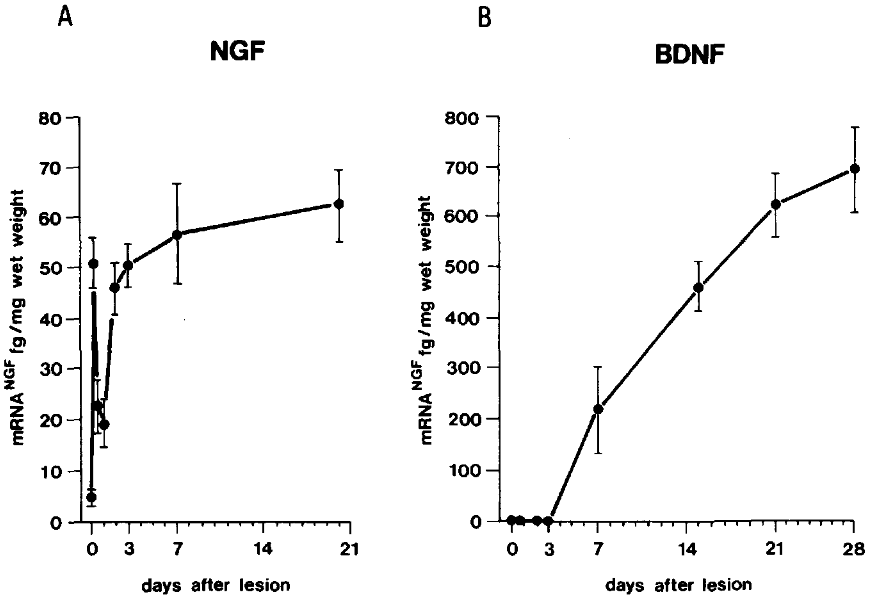

FIG. 29-2. Comparison of the time course of mRNA induction for nerve growth factor (*NGF*) and brain-derived neurotrophic factor (*BDNF*) mRNA in the distal segment of the sciatic nerve during the first weeks after lesion. [From Meyer et al. (1992), with permission.]

by macrophages invading the lesioned nerve (Heumann et al., 1987b; Lindholm et al., 1987). Interestingly, the increase of NGF mRNA is not restricted to nerves that contain the nerve fibers of NGF-dependent sensory or sympathetic neurons. For example, it occurs also in the lesioned facial nerve, which contains only the axons of NGF-independent motoneurons (Heumann et al., 1987a).

NGF is not the only neurotrophin that is upregulated after nerve lesion. BDNF mRNA is also increased up to high levels at the lesion site and the distal part of the lesioned nerve (Meyer et al., 1992). However, the increase of BDNF mRNA is much slower than that of NGF mRNA. It was not detectable before the second half of the first week after lesion, and increased continuously during the first 4 weeks after lesion. One month after lesion, BDNF mRNA levels were 10 times higher than the levels of NGF mRNA, and a plateau was not reached up to that time (Figure 29-2). The massive and long-lasting increase of BDNF mRNA in rat peripheral nerves after lesion might be of considerable significance to BDNF-dependent sensory and motor neurons during regeneration.

Ciliary Neurotrophic Factor

Ciliary neurotrophic factor (CNTF) has originally been identified as an activity present in eye extracts, which supports the survival of embryonic chick ciliary neurons in culture (Helfand et al., 1976). Subsequent studies with semipurified CNTF showed that this neurotrophic factor differed significantly from the neurotrophins by its broad spectrum of responsive neurons of the embryonic chick. CNTF not only supports ciliary neurons, but also sympathetic, sensory (Barbin et al., 1984), nodose, trigeminal, and, in particular, motoneurons (reviewed in Sendtner et al., 1991). Moreover, CNTF has also been shown to act on glial precursor cells of the oligodendrocyte lineage (Lillien and Raff, 1990) and to support the survival of differentiated oligodendrocytes in culture (Louis et al., 1993). The final purification and subsequent cloning of the cDNA (Lin et al., 1989; Stöckli et al., 1989) of CNTF showed that this factor is not structurally related to the neurotrophins. In particular, CNTF lacks a hydrophobic leader sequence, which is typical of secretory proteins such as the neurotrophins. It is not yet known how CNTF is made available by cells where it is produced in order to act on its responsive neurons.

Regulation of Ciliary Neurotrophic Factor Expression. In addition to these specific properties, the temporal and spatial expression of CNTF mRNA and protein differs distinctly from that of the neurotrophins. In the developing rat, CNTF expression is absent during the time periods when neurons differentiate or when a significant proportion of postmitotic peripheral neurons undergo cell death (Stöckli et al., 1991). Therefore it was assumed that CNTF does not play an essential function during

these developmental stages. In the rat, the first expression of CNTF is detectable in Schwann cells of the peripheral nerves at postnatal day 4, the time when Schwann cells stop dividing and myelination starts (Stöckli et al., 1989). In the adult peripheral nervous system, the very high amounts of CNTF in peripheral nerves are localized in the cytoplasm of myelinating Schwann cells (Stöckli et al., 1991; Rende et al., 1992). It is still not clear whether CNTF can be released from unlesioned Schwann cells or whether release, and thus availability, for responsive neurons occurs only after nerve lesion. After peripheral nerve lesion in the adult rat, CNTF mRNA is rapidly downregulated in the distal stump. However, CNTF protein, biological activity, and immunoreactivity are still detectable (Friedman et al., 1992; Sendtner et al., 1992b). One week after lesion, CNTF protein levels are still 1000 times higher than the levels of NGF (Table 29-1), whose expression is upregulated after lesion. Thus it seems plausible that CNTF might play a role in maintaining survival of lesioned motor and sensory neurons, at least during the early stages after peripheral nerve lesion.

The Ciliary Neurotrophic Factor Receptor Complex. A specific low-affinity receptor component for CNTF has been identified and cloned (Davis et al., 1991). This molecule, which lacks an intracellular and a transmembrane region, is anchored to the membrane via glycosylphophatidylinositol (GPI) anchor. Recently, evidence has been presented that two further transmembrane molecules are necessary for CNTF signal transduction: the signal transducing subunit of the IL-6 receptor complex, gp130 and the LIFRβ (reviewed by Taga and Kishimoto, 1992, Ip and Yancopoulos, 1992). The low-affinity CNTFRα also exists in a soluble form, which has been detected in cerebrospinal fluid and in serum of adult humans (Davis et al., 1993). This indicates that CNTF-responsive cells do not necessarily depend on endogenous CNTFRα expression (Figure 29-3). In the presence of soluble CNTFRα, the expression of gp130 and LIFRβ is sufficient to render cells responsive to CNTF. If this also occurs under physiological conditions *in vivo,* then the spectrum of CNTF responsive cells would go far beyond the neuronal cells which have been shown to express CNTFRα (Ip et al., 1993a).

The Fibroblast Growth Factor Gene Family

Fibroblast growth factors are a large family of growth factors with actions on a great variety of cells (for review see Klagsbrun, 1989; Goldfarb, 1990). Similar to CNTF, the prototypic FGFs—acidic FGF (FGF1) and basic FGF (FGF2)—lack a signal peptide sequence. Thus it is not known how these molecules are released from synthesizing cells in order to exert their physiological function. In contrast, the other six known members of the FGF gene family are secreted from synthesizing cells.

Binding of Fibroblast Growth Factors to Heparan Sulfate Proteoglycans. In contrast to the known neurotrophins and to CNTF, the members of the FGF gene family show affinity (in the range of 10^{-7} to 10^{-9} mol) to the glycosaminoglycan heparin (Yayon et al., 1991; for review see Givol and Yayon, 1992; Partanen et al., 1992). This has led to the identification of extracellular matrix and cell surface-bound heparan sulfate proteoglycans (HSPG) as a class of physiological binding sites for these molecules. HSPGs are ubiquitous macromolecules with a high degree of structural variability, whose composition is strongly regulated both during development and in a tissue specific manner (for review see Bernfield

TABLE 29-1. *Comparison of the Levels of NGF, CNTF, and FGF1 Biological Activity in the Sciatic Nerve of the Adult Rat After Lesion*

	NGF (trophic units/mg protein)	CNTF (trophic units/mg protein)	FGF1 (mitotic units/mg protein)
Unlesioned sciatic nerve	0.6 ± 0.2[a]	17500 ± 2500[b]	768 ± 67[c]
Distal sciatic nerve 7 days after lesion	2.5 ± 0.6[a]	3150 ± 620[b]	9 ± 4[c]

Data are modified according to Heumann et al. (1987), Sendtner et al. (1992a), and Eckenstein et al. (1991).

NGF, nerve growth factor; CNTF, ciliary neurotrophic factor; FGF1, fibroblast growth factor-1.

[a]One trophic unit of NGF corresponds to about 75 pg/ml, which is the amount necessary for stimulating half-maximal survival of embryonic day 8 chick dorsal root sensory neurons in culture.

[b]One trophic unit of CNTF corresponds to about 30 pg/ml, the amount that supports half maximal survival of embryonic day 8 chick ciliary neurons in culture.

[c]One mitotic unit of FGF1 corresponds to about 150 pg/ml which induces half-maximal stimulation of mitosis in cultures of AK12-2B cells.

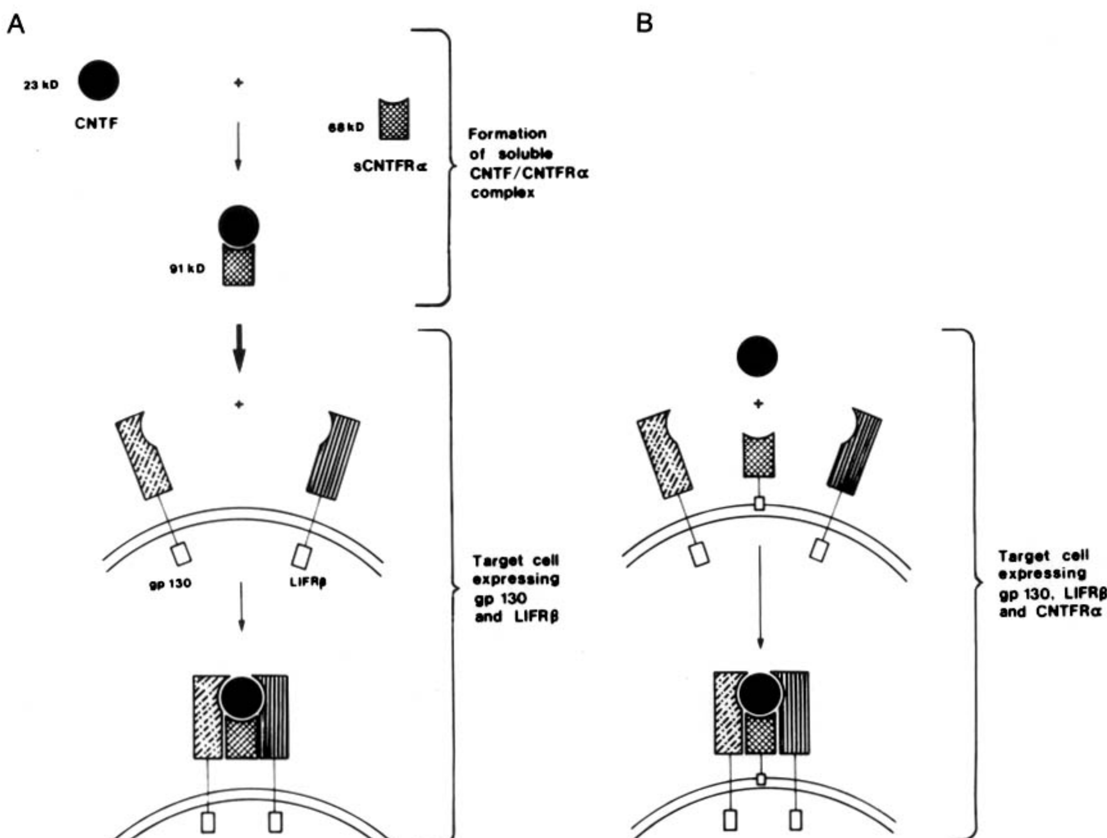

FIG. 29-3. Model of ciliary neurotrophic factor association with its receptor components. (A) After association with the soluble CNTFRα in body fluids (blood, cerebrospinal fluid) CNTF can act on any cell expressing gp 130 and LIFRβ. (B) In neuronal cells, all three components of the CNTF receptor are expressed so that the association with a soluble CNTFRα is not necessary for CNTF action.

et al., 1992). Recently it has been shown that the specificity of the major embryonic forms of HSPG in developing neuroepithelium changes during development from a form with relative specificity for FGF1 to another form that preferentially recognizes and potentiates the mitotic actions of FGF2 (Nurcombe et al., 1993). The specificity of HSPG for different members of the FGF gene family and the resulting implications for the physiological functions of the FGFs are still poorly understood. The basal membranes of peripheral nerves and target tissues, such as skeletal muscle, bind different members of the FGF gene family (Vaca et al., 1989; Hughes et al., 1993; for review see Klagsbrun, 1989; Goldfarb, 1990). It is an intriguing possibility that the composition of these basal membranes can modify the actions of different members of the FGF gene family on responsive neurons and nonneuronal cells within the peripheral nervous system.

Similarly, nothing is known so far about the molecular nature of HSPG on the cell surface of glial cells and neurons. Analysis of the conditions required for high-affinity binding of FGF2 to FGF receptors has revealed the importance of the HSPG, which, together with FGF2 as a complex, binds and activates FGF receptors (Yayon et al., 1991). Thus, the responsiveness of different cells to the FGFs could not only be dependent on the expression of specific FGF receptors, but also on the availability of the specific HSPGs. A variety of different FGF receptors has been cloned so far from avian and mammalan sources, and some types of FGF receptor mRNAs have in the meantime also been identified in central and peripheral neurons (Wanaka et al., 1990; see also reviews of Partanen et al., 1992; Givol and Yayon, 1992).

Functions and Expression of Fibroblast Growth Factors in the Peripheral Nervous System. In spite of many studies on the function of FGF1 and FGF2 on cultured neuronal and nonneuronal cells, not much is known on the physiological function of the FGFs in the peripheral nervous system. FGF1 and FGF2 have so far been identified as survival factors for cultured ciliary and motoneurons (Unsicker et al., 1987, 1992a, 1992b; Arakawa et al., 1990). Recently also

FGF3 (K-FGF) and FGF5 were shown to be active on motoneuron survival (Hughes et al., 1993). At least for FGF2, survival of ciliary and motoneurons has been shown to be supported in single-cell cultures (Sendtner et al., 1991; Unsicker et al., 1992a), demonstrating that the effect of FGF2 on these neurons is direct and not mediated by other cells in the peripheral nervous system. Moreover, FGF1 and FGF2 can induce mitosis of cultured Schwann cells (Davis and Stroobant, 1990), which suggests a further role of the members of this gene family in the peripheral nervous system.

FGF1 is expressed in very high quantities in neurons, particularly in motoneurons, and FGF1 protein is detectable in axons of peripheral nerves (Eckenstein et al., 1991). After lesion, FGF1 protein and biological activity levels are rapidly decreased in extracts from the distal parts of the lesioned nerves (see Table 29-1). This does not fit with a role as a lesion factor for the injured neurons, as has been suggested for CNTF.

It has been suggested that FGF2 is expressed in skeletal muscle and might act as a target derived neurotrophic factor for spinal motoneurons (Vaca et al., 1989). However, the only evidence presented was the molecular size of semipurified activity (about 18 kD) and the affinity to heparin, two parameters that are shared by FGF-5. In contrast to FGF-2, significant levels of FGF-5 mRNA are found in developing and adult skeletal muscle both by *in situ* hybridization techniques and Northern blot analysis (Haub and Goldfarb, 1991; Hughes et al., 1993). In addition, immunoprecipitation studies have shown that FGF-5 is the major motoneuron survival activity from embryonic rat skeletal muscle extracts for cultured chick motoneurons. In summary, future studies have to show how the FGFs are involved in the complex interactions between target tissues, Schwann cells, and neurons within the peripheral nervous system.

Insulin-like Growth Factors

IGF-I and IGF-II are synthesized by a variety of tissues, including the liver, pituitary, and nervous system, and are present in relatively high concentrations in serum and cerebral fluid (Recio-Pinto et al., 1986). During the last few years, evidence has increased, suggesting that these factors could play a physiological role in the neuromuscular system: They have been shown to promote neurite outgrowth from sympathetic and sensory neurons, and IGF-I is retrogradely transported in the sciatic nerve of the adult rat (Hansson et al., 1987). Furthermore, embryonic chick (Arakawa et al., 1990) and rat motoneurons in culture can survive and grow neurites in the presence of IGF-I and IGF-II, and these cells express high-affinity binding sites for these molecules on their cellular processes (Caroni and Grandes, 1990). The injection of IGF-II on the surface of the gluteus muscle of the adult rat induces nodal sprouting of innervating nerve fibers and terminal sprouting at the endplates (Caroni and Becker, 1992). During development both IGF-I and IGF-II mRNA expression in muscle are sharply decreased at the onset of the developmental period of synapse elimination, and the mRNAs for both factors are reexpressed in skeletal muscle after denervation.

GAP-43 and tubulin-α_1 expression in motoneurons, which are also both decreased during synapse elimination, can be maintained at significant levels by the local addition of exogenous IGF-I during the period of synapse elimination. The increase of IGF-I mRNA after muscle denervation in adult animals appears to occur more rapidly than that of IGF-II, and both muscle fibers and interstitial cells might be the source of these factors 1 week after muscular denervation. In addition to the increased IGF expression in muscle, IGF-I receptor mRNA expression is induced in the corresponding spinal motoneurons. It can be concluded from these results that elevated levels of IGFs in denervated muscle, and probably also in distal parts of lesioned nerves, might trigger coordinate regenerative reactions, which lead to nerve sprouting under pathophysiological conditions.

Cooperative Actions of Target- and Glial-Derived Neurotrophic Factors on Spinal Motoneurons

The skeletal musculature is innervated by motoneurons whose cell bodies reside in either the ventral spinal cord or in brainstem motor nuclei. The peripheral nerves consist to a considerable part of motoneuron axons, and the loss of motor function appears as the predominant impairment after damage of peripheral nerves. Thus the identification of the growth factors that support survival and regeneration of adult motoneurons is of particular clinical relevance.

The establishment of highly enriched cell cultures of embryonic chick spinal motoneurons has helped to identify a variety of neurotrophic molecules and growth factors, which can keep these cells alive in culture. Such factors include CNTF, FGF2, FGF5, and, to a lesser extent, FGF1, FGF3, and IGF-I (Arakawa et al., 1990; Hughes et al., 1993). Leukemia inhibitory factor (LIF), which is structurally related to CNTF (Bazan, 1991) and shares at least two re-

ceptor subunits (gp 130 and LIFRβ) with CNTF (Ip and Yancopoulos, 1992), has been identified as a survival factor in rat motoneuron cultures (Martinou et al., 1992). At least some of these factors are also active in supporting motoneuron survival *in vivo,* both during embryonic development (Oppenheim et al., 1991, 1992b; Nurcombe et al., 1991), and after peripheral nerve lesion in newborn rats (Sendtner et al., 1990, 1992a; Yan et al., 1992; Koliatsos et al., 1993). In particular, CNTF, BDNF, and NT-3 have been shown to be active. Interestingly, FGF2 did not show significant survival effects on motoneurons under the same experimental conditions where CNTF and BDNF were active (Nurcombe et al., 1991; Oppenheim et al., 1992a).

The positive effects of BDNF, NT-3, and NT-4/5 on motoneuron survival seem to be direct effects, as isolated and highly enriched embryonic rat motoneurons in culture can survive in the presence of either BDNF, NT-3, or NT-4/5 (Henderson et al., 1993). It has also been shown that these cells express both *trk* B and *trk* C in cell culture and *in vivo* (Henderson et al., 1993), which suggests that the survival effects are mediated via these specific receptor molecules. These data show that molecules of the neurotrophin gene family are also capable of supporting motoneuron survival *in vivo* in a similar manner to ciliary neurotrophic factor.

In contrast to CNTF, however, both NT-3 and BDNF are expressed during embryonic development in specific regions both of the central and peripheral nervous system and in the periphery. Both in the developing and in the adult rat, significant quantities of BDNF mRNA have been detected in the target tissues of spinal motoneurons, namely, skeletal muscle. In comparison to BDNF, the effect of NT-3 seems quite small, and it is not clear whether NT-3 supports only a small subpopulation of motoneurons. Neurotrophin-3 mRNA has been reported to be expressed at high levels in the rat spinal cord between embryonic days 13 and 16 (Ernfors and Persson, 1991). Thereafter, expression decreases gradually to barely detectable levels at birth. Using *in situ* hybridization techniques, the spinal motoneurons themselves have been identified as the site of synthesis of NT-3. It is not clear at the moment whether endogenous production of NT-3 in motoneurons could act in an autocrine manner during development, or whether only postnatally do the relatively high quantities of NT-3 produced in muscle contribute to the target-derived neurotrophic input to motoneurons during later development. Further studies, such as gene-targeting experiments, have to be carried out in order to judge the functional relevance of NT-3 for developing postnatal motoneurons.

The recent observation that BDNF expression is upregulated in the sciatic nerve of the adult rat after lesion (Meyer et al., 1992), is particularly interesting when these results are compared to those of CNTF. CNTF, which we have found to be highly active in supporting survival of lesioned facial motoneurons (Sendtner et al., 1990), is expressed in high amounts in myelinated Schwann cells of peripheral nerves of the adult rat. After lesion, significant quantities of CNTF protein seem to persist for at least 1 week in the lesioned nerve. Immunohistochemistry at the lesion site and the distal nerve segment has shown that CNTF is present in the extracellular space and it seems to be available to regenerating axons (Figure 29-4). The expression of BDNF starts with a delay of at least 3 days after lesion of the sciatic nerve (Figure 29-2), and there is a gradual increase up to the third and fourth week after lesion. Thus, after nerve lesion, motoneurons are supported first by CNTF released from injured Schwann cells, and then by BDNF at later postinjury stages, and BDNF would then support the regeneration of motoneurons to the periphery. The observation that BDNF and CNTF can significantly support the survival of motoneurons after lesion in postnatal animals indicate that these factors could have important functions on motoneurons, and also mediate the regeneration to the periphery after neuronal lesion.

NEURON-DERIVED GROWTH FACTORS FOR PERIPHERAL GLIAL CELLS

In vertebrates, not only do nonneuronal cells provide growth or trophic factors for survival and functional maintenance of neurons, but also regulatory interactions between neurons and nonneuronal cells have been identified. For example, the capability of amphibians to regenerate limbs after amputation is nerve-dependent, and it is believed that soluble factors from neurons play an essential role in this process. Also, in higher vertebrates where such dramatic regenerative events do not occur, nerve cells seem to play a major regulatory role both for developing and the adult Schwann cells. Schwann cell precursor cells from embryonic rat sciatic nerves cannot survive in culture without soluble proteins, which are provided from neurons. Although the responsible molecules are not identified so far, it has been shown that members of the FGF gene family are active in supporting the survival of cultured Schwann cell precursors (Jessen and Mirsky, 1992).

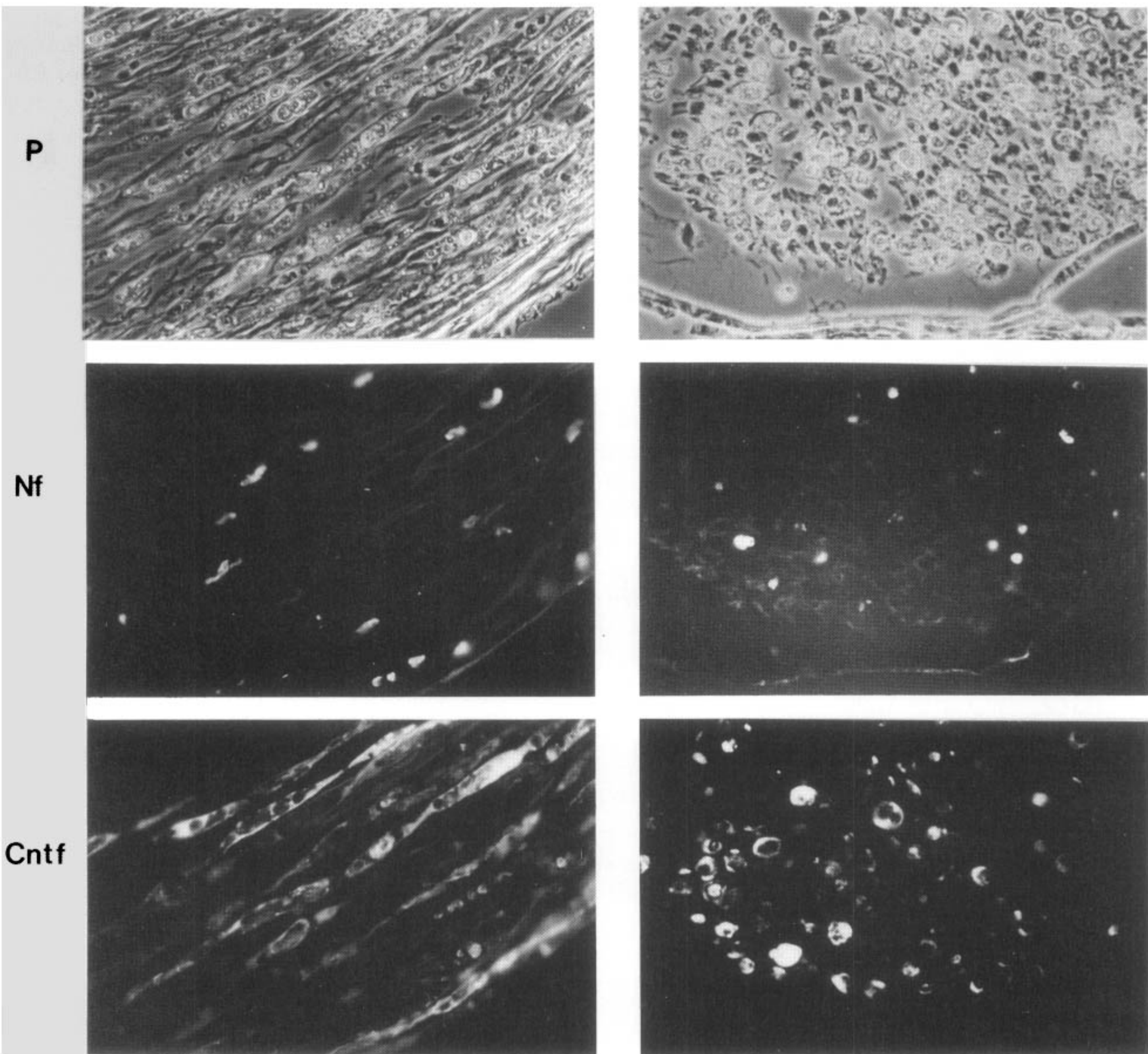

FIG. 29-4. Immunolocation of ciliary neurotrophic factor in sections of the sciatic nerve 6 days after transsection. (*Left*) Longitudinal section. (*Right*) Transverse section. Neurofilament and CNTF immunoreactivity were detected in the same sections by double-labeling techniques as described in Sendtner et al. (1992b). P, phase contrast; Nf, neurofilament immunohistochemistry; Cntf, ciliary neurotrophic factor immunohistochemistry. Six days after sciatic nerve transsection in the adult rat when most of the axons are degraded, CNTF immunoreactivity is still detectable in association with myelin breakdown products, basal membranes, and at extracellular locations where neurofilament staining indicates axonal remnants.

Glial Growth Factor

Postnatally, differentiated Schwann cells usually do not divide in peripheral nerves of higher vertebrates. However, after nerve injury, they dedifferentiate and proliferate rapidly. It has been proposed for a long time that neuron-derived mitogens are involved in this process. Evidence was presented as early as 1978 (Raff et al.) that bovine brain and pituitary extracts contain soluble factors that stimulate the mitosis of Schwann cells in culture. It has been deduced from these experiments that neuronal cells might be capable of synthesizing such factors and thus are able to exert control over cell division and differentiation of these glial cells during development. However, only recently has the molecular nature of these factors from bovine brain and pituitary gland been identified (Marchionni et al., 1993). The glial growth factors are identical with the recently

identified ligands (Wen et al., 1992) for the p185 *erb* B2 tyrosine kinase (also known under the name *neu* oncogene). A transformed version of this protooncogenic tyrosine kinase receptor, has originally been identified as a dominant transforming gene in neurogliomas and schwannomas after experimental tumor induction with ethylnitrosourea in rats (Kokai et al., 1987; also summarized in Wen et al., 1992).

The following pieces of evidence have now been presented which argue for a physiological involvement of glial growth factors and the corresponding p185 *erb* B2 tyrosine kinase receptor in the interactions between neurons and glia:

1. Recombinant glial growth factors can stimulate the mitosis of isolated Schwann cells in culture. There are both membrane bound forms of the active factors, which are encoded by splice variants with a cytoplasmic and transmembrane domain, and soluble splice variants that lack cytoplasmic and transmembrane domain.
2. The receptor for these ligands, the p185 *erb*B2 tyrosine kinase molecule, is physiologically expressed by Schwann cells during development, and is upregulated during Wallerian degeneration (Cohen et al., 1992). Furthermore, this molecule is autophosphorylated upon exposure of cultured Schwann cells to different splice variants of glial growth factor, indicating that p185 *erb*B2 indeed could transduce the signal upon binding of its corresponding ligands.
3. Glial growth factors are expressed by motoneurons in the developing spinal cord and by sensory neurons of the dorsal root ganglia of the mouse at the time when the axons of these neurons grow out within the prospective peripheral nerves, which coincides with the beginning of the period when the mitosis rates of Schwann cells in peripheral nerves are highly increased (reviewed by Jessen and Mirsky, 1992).

However, the expression of glial growth factor expression is maintained for a prolonged period. It seems likely that these molecules also trigger other features of Schwann cell differentiation, such as ensheathement of axons and myelination. Indeed, the function of these molecules are not restricted to the interactions between neuronal and glial cells. At the same time when the ligands for *erb*B2 were identified to act on Schwann cells, the same molecules were found to exert another important function within the peripheral nervous system, that is, the potentiation of acetylcholine receptor expression in skeletal muscle. The activity in chick brain described first in the late 1970s, which induces acetylcholine receptor expression in cultures of myotubes or muscle cells, is identical to glial growth factor (Corfas et al., 1993; Falls et al., 1993).

The analysis of the biological function of the *erb*B2 ligands has just begun, and it appears that the results described here represent only the beginning of the understanding of how neuronal cells regulate properties of Schwann cells and their target cells in the peripheral nervous system on the molecular level.

CONCLUSION

In summary, a variety of different neurotrophic and growth-promoting factors have been identified at the molecular level, which contribute to the regulation of cell-cell interaction in the peripheral nervous system. Most of these factors were identified by their effects on cultured cells *in vitro*, and it is a focus of present research to analyze the physiological function of each factor *in vivo*. Evidence is increasing that different molecules might act together to promote complex functions such as the regeneration of lesioned nerve fibers. New techniques such as gene targeting by homologous recombination, or the establishment of transgenic animals that overexpress the genes of such factors, under the direction of tissue-specific promoters, might help in the future to elucidate the roles of these molecules in the functional maintenance of the peripheral nervous system.

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30 Growth factors and their receptors in the central nervous system

G. LABOURDETTE AND M. SENSENBRENNER

GROWTH FACTORS AND THEIR RECEPTORS

Nerve Growth Factor and Epidermal Growth Factor

The first growth factor to be characterized was discovered by Levi-Montalcini and coworkers. In their *in vitro* investigations these authors showed that a soluble extract of mouse sarcoma cells stimulated fiber outgrowth from explanted sympathetic and dorsal root ganglia. The soluble factor responsible for this effect was called *nerve growth factor* (NGF) and was subsequently found in high concentration in adult male mouse submaxillary gland (review by Levi-Montalcini, 1987). Later, a second growth factor, the *epidermal growth factor* (EGF) was detected also in the mouse submaxillary gland, on the basis of its stimulatory effect on the development of the epidermis of mouse embryos *in vivo*. Many other growth factors have been characterized since then and most of them have been shown to occur in brain.

Classification and Mode of Action of the Growth Factors

Growth factors are polypeptides synthesized by most tissues that contribute to the control of cell developmental processes like proliferation, differentiation, survival, migration, and maturation and that act usually by binding to membrane receptors. In most cases they elicit various (pleiotropic) effects in a wide variety of cells. A classification of the growth factors related to glia is proposed in Table 30-1 and Table 30-2. The ubiquitous growth factors act on and are found in many cells, and are actually families of factors. They are shown in Table 30-1, with the number of amino acids found in the more mature forms and the names of their receptors when they are known. Other growth factors related to glia have been classified in Table 30-2.

In the brain, before the occurrence of the blood-brain barrier (a few days after birth in rat) growth factors from the bloodstream can act on nerve cells. Afterward, the growth factors are produced endogenously.

The various classes of structure of the growth factor receptors are schematically shown in Figure 30-1. Most growth factor receptors are monomeric and have a tyrosine kinase activity in their intracellular domain, such as fibroblast growth factor receptors (FGFRs) (Fantl et al., 1993). Receptors of insulin-like growth factor-1 (IGF-1) and insulin have a heterotetrameric structure. Transforming growth factor β (TGFβ) receptors I and II have a serine-threonine activity in their cytoplasmic domain. Some other receptors do not have an enzymatic activity; they can elicit their effects through an interaction with G proteins, such as tumor necrosis factor-αR (TNFαR) or thrombin-R, or through the formation of complexes with other receptors, such as interleukin-6Rα (IL-6Rα), which has a very small cytoplasmic domain, or ciliary neurotrophic factor-Rα (CNTFRα), which is anchored to the plasma membrane via a glycosylphosphatidylinositol linkage. It seems that, in order to be activated, most, if not all, growth factor receptors must form complexes before or after the binding of the ligands (Figure 30-2). Glycosaminoglycans have been reported to be necessary for FGFRs and possibly for TGFβRs activation, probably by presenting the ligands to the enzymatic receptors. Some high-affinity receptors are formed by the association of two or more receptors, which alone have either a low affinity or no affinity for the ligand, as shown for IL-6R, CNTFR, or leukemia inhibitory factor receptor (LIFR) (Miyajima et al., 1992).

BIOLOGICAL SYSTEMS FOR GROWTH FACTOR STUDIES

The *in vivo* investigations include work on tissue extracts, on tissue slices, and (by injection) on the living organism of normal or of transgenic animals. *In vitro*, different kinds of tissue cultures can be used, either primary cultures or cell lines. Primary cultures

TABLE 30-1. *Ubiquitous Growth Factors*

Factors	Full Names	Amino Acids	Receptors
EGF family			
EGF	Epidermal growth factor	53	EGFR
TGFα	Transforming growth factor α	50	EGFR
AR	Amphiregulin	78	EGFR
SGDF	Schwannoma-derived growth factor	(35)	EGFR
HB-EGF	Heparin-binding EGF-like growth factor	(22)	EGFR
HRGα/NDF	Heregulin/NEU differentiation factor	(45)	EGFR2/ErbB2
FGF family			
FGF-1 (aFGF)	(acidic) Fibroblast growth factor	140	FGFR1, -2, -3, -4
FGF-2 (bFGF)	(basic) Fibroblast growth factor	154	FGFR1, -2, -3
FGF-3 (int-2)		231	
FGF-4 (hst-1 or K-FGF)	Human stomach tumor gene-1 or Kaposi sarcoma FGF	148	FGFR1, -2
FGF-5		267	
FGF-6 (hst-2)	hst-1 related gene	208	
FGF-7 (KGF)	Keratinocyte growth factor	163	FGFR2
IGF family			
IGF-1	Insulin-like growth factor	70	IGF-1R
IGF-2		67	IGF-2R
Insulin		51	IR
PDGF family			
PDGF-AA	Platelet-derived growth factor	2×125	PDGFRα
PDGF-BB		2×114	PDGFRβ
PDGF-AB		114+125	PDGFRα-β
TGF-β family			
TGFβ1	Transforming growth factor β	2×112	TGFβRI, -II, -III
TGFβ2		2×112	TGFβRI, -II
TGFβ3		2×112	TGFβRI, -II, -III

TABLE 30-2. *Other Growth Factors Related to Glia*

Miscellaneous factors and active peptides	
	Thrombin
PA	Plasminogen activator
PD-ECGF	Platelet-derived endothelial cell GF
GMF	Glia maturation factor
ET-(1-3)	Endothelin (1 to 3)
VIP	Vasointestinal peptide
Neurotrophic factors	
NGF	Nerve growth factor
BDNF	Brain-derived neurotrophic factor
NT-(3-5)	Neurotrophin (3 to 5)
GDNF	Glia-derived neurotrophic factor
CNTF	Ciliary neurotrophic factor
CDF/LIF	Cholinergic differentiation factor or leukemia inhibitory factor
GDN	Glia-derived nexin
Cytokines	
IL-(1-13)	Interleukins
TNF(α/β)	Tumor necrosis factor
BCGF	B-cell growth factor
IFN(α-γ)	Interferon
Hematopoietic factors	
M-CSF	Macrophage CSF (or CSF-1)
GM-CSF	Granulocyte and macrophage CSF
G-CSF	Granulocyte CSF
IL-3	Multi-CSF or interleukin-3

CSF, colony stimulating factor

include explants, reaggregates, or dissociated monolayer cultures derived from embryos or young animals.

Astroblasts and Ependymal Cells in Monolayer Culture

Glial cell cultures are usually derived from brains of newborn rats. In most studies, astroblast (type 1) cultures have been obtained by growing the dissociated cells of newborn rat brain in a culture medium enriched with 10% fetal calf serum (for review see Sensenbrenner et al., 1986). These cells proliferate for 7 to 10 days and then become confluent as a monolayer of large flat cells. They mature as a function of time. For example, expression of glial fibrillary acidic protein (GFAP), S-100 protein, and glutamine synthetase (GS) begins after a few days *in vitro* and increases roughly between days 10 and 30 *in vitro*.

Cultures highly enriched in ependymal cells can be obtained from the same dissociated cells, but grown

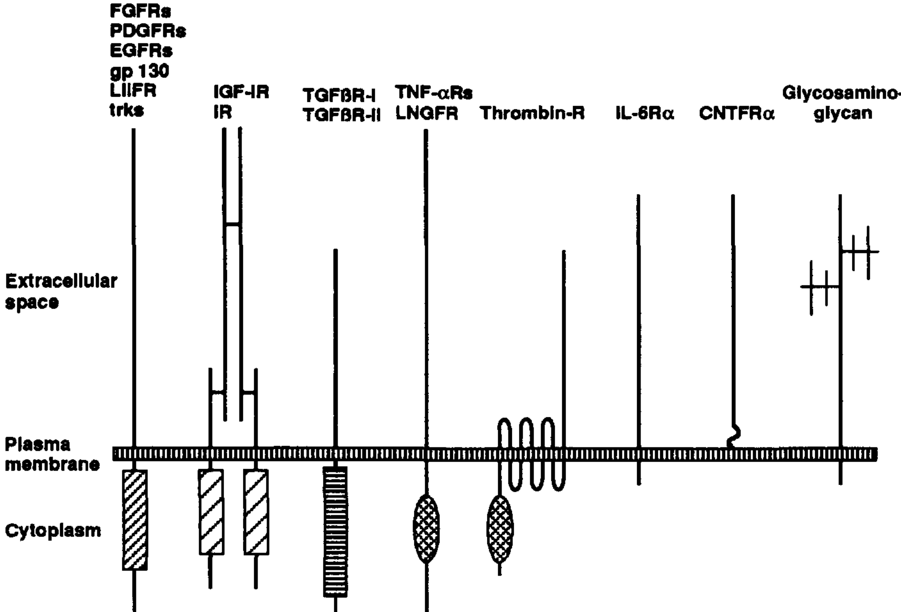

FIG. 30-1. Growth factor receptors and receptor complexes. In the cytoplasmic compartment, *obliquely hatched boxes* represent tyrosine kinase domains, the *horizontally hatched box* represents a serine-threonine kinase domain, the *ovals* represent domains interacting with G proteins.

in the absence of serum and in the presence of thrombin (Weibel et al., 1986).

Oligodendrocytes and Their Progenitors in Monolayer Mixed Culture

If dissociated cells from newborn rat brain are grown at high cell density and in the presence of 10% calf serum, small round cells appear on the monolayer of flat astroblasts (type 1) (Labourdette et al., 1979). They proliferate actively for about 2 weeks and then differentiate into oligodendrocytes and express progressively, over a period of 4 to 7 weeks, the various myelin markers: sulfatides, galactocerebrosides, cyclic nucleotide phosphohydrolase (CNP), myelin basic proteins (MBP), carbonic anhydrase type II, proteolipid protein (PLP), ceramide galactosyl sulfotransferase, ceramide sulfotransferase, and myelin-associated glycoprotein. Cultures derived from rat embryo optic nerve are enriched in oligodendrocytes and have been also extensively studied.

Glial Progenitor Cells in Pure Culture and Type 2 Astrocytes

The small, round cells described above can be detached selectively from the mixed glial culture by flushing culture medium on the cell layer or by rotary agitation for 16 to 24 hours. After about 10 days in such primary cultures the small cells are mostly bipotential precursor cells (also called O-2A progenitor cells), characterized by their reaction with the A2B5 monoclonal antibody. In secondary culture, they can differentiate either into oligodendrocytes if they are grown in the absence of serum, or into type-2 astrocytes in the presence of 20% fetal calf serum (Raff et al., 1983).

Oligodendrocytes

Oligodendrocytes can be obtained in culture either by the differentiation, in the absence of serum, of O-2A progenitor cells isolated as described in the previous section, or by the selective detachment of already mature cells from an aged mixed glial culture (after 30 days *in vitro* or more). They can also be obtained from brain of adult animals by an enzymatic dissociation of the cells, followed by a separation of the various cell types by centrifugation in a gradient of sucrose or of Percoll.

Brain Cell Reaggregates

Cells dissociated from fetal rats (usually 15-day-old), can form aggregates when they are maintained in flasks under constant rotary agitation (Honegger and Tenot-Sparti, 1992). In this culture system, cells are surrounded by a microenvironment resembling

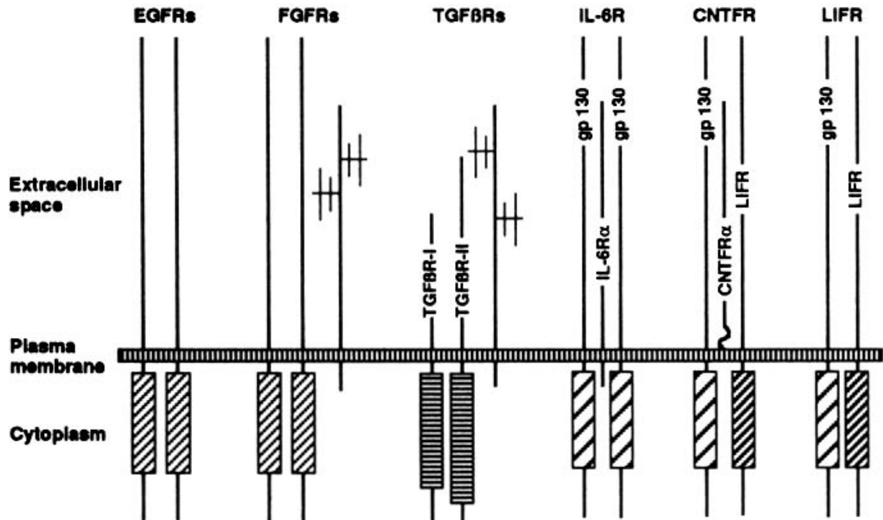

FIG. 30-2. Growth factor receptor complexes. Same specifications as in Figure 30-1.

that found *in vivo,* but which can be manipulated to some extent by modifying the culture medium.

Immortalized and Transformed Glial Cells

When primary cultures were not available cell lines derived from normal or transformed cells have been used. For instance, the C6 glioma cell line, derived from a nitrosourea-induced tumor, has been used as a model of astrocytes and of oligodendrocytes and the spontaneously immortalized CG4 cell line is used as a model of O-2A progenitor cells (Louis et al., 1993).

In glial tumors, the cells are not homogeneous. They can be grown as a mixture or they can be cloned to obtain only one tumoral cell type. Such cells are used mainly to study the mechanisms of transformation and the possible involvement of growth factors.

FIBROBLAST GROWTH FACTORS

On the basis of the hypothesis that soluble factors should participate in the development of cells of the central nervous system (CNS), it has been shown that soluble brain extracts from chick embryos are able to stimulate the morphological differentiation and the proliferation of astroblasts from chick embryos in dissociated primary culture (Sensenbrenner et al., 1972). Later on, acidic and basic fibroblast growth factor (aFGF and bFGF, also called FGF-1 and FGF-2, respectively) have been found to be the active molecules present in these soluble brain extracts (Pettmann et al., 1985).

Family Relationships

The two first identified fibroblast growth factors, aFGF and bFGF, have been purified to homogeneity from bovine pituitary gland and brain. They are composed of a single polypeptidic chain and share 55% amino acid sequence identities.

Five other structurally related FGFs (Table 30-1), with 35 to 55% sequence homology have been identified (for review see Baird and Klagsbrun, 1991). They differ from aFGF and bFGF by the presence of a signal peptide, glycosylation sides, expression only during embryonic development, and specific target cells.

Receptor Family

To date, a group of four transmembrane high-affinity receptors have been identified and designated as FGFR1, R2, R3, and R4 (for review see Partanen et al., 1992). The members of the FGF family have overlapping affinities for these receptors (Table 30-1).

Another class of binding sites for FGFs is related to heparin-like glycosaminoglycans (GAGs) and heparan sulfate proteoglycans (Figure 30-1). They have been shown to be required for bFGF and K-FGF to bind its high-affinity receptors (for review see Partanen et al., 1992) (Figure 30-2).

Cellular Localization in the Central Nervous System In Vivo *and* In Vitro

Acidic FGF and bFGF are widely and abundantly expressed in the developing and adult CNS of chick, mouse, rat, monkey, and human. FGF-5 is also pres-

ent in the central nervous system (Table 30-3). Most immunocytochemical staining experiments have revealed that aFGF and bFGF are associated with neurons *in vivo* and *in vitro*. However, aFGF, bFGF, and their mRNAs have also been found in astroglial cells (Tables 30-3 and 30-4). Acidic FGF has been localized in a subpopulation of ependymal cells and tanycytes, as well as in some glial cells of adult rat brain (Tooyama et al., 1991). In culture, rat and bovine brain astrocytes as well as mouse cerebellum astrocytes synthesize bFGF and to a much lesser extent also aFGF (Table 30-4). Several authors pointed out a nuclear localization of bFGF in astrocytes *in vivo* as well as *in vitro* (Cintra et al., 1991; Woodward et al., 1992) and of aFGF in oligodendroglial cells *in vivo* (Tourbah et al., 1991).

Although aFGF and bFGF lack a signal peptide sequence, a recent study has clearly indicated that bFGF-like peptides are released from both glial and neuronal cells *in vitro* (Araujo and Cotman, 1992). Furthermore, this release is differentially regulated by several lymphokines and other growth factors.

Cellular Localization of Receptors in the Central Nervous System and in Neural Cells In Vitro

The presence of high-affinity receptors for FGF has been reported in the CNS of adult bovine, rat, and guinea pig. FGF receptor mRNA is present in the developing chick, mouse, and rat brain. The expression of FGF receptor mRNA *in situ* has been found

TABLE 30-3. *Growth Factors Normally Present in the Central Nervous System*

Factors	Methods used	Species	Main zone(s)	Age	Expression in: Neur	Astr	Epen	Ref.
aFGF	H, I	Rat, Hu	All	D,A	+	+	+	Tooyama et al., 1991; Wilcox and Unnerstall, 1991
bFGF	H, I	Rat, Hu	All	D,A	+	+		Janet et al., 1987; Pettmann et al., 1986; Woodward et al., 1992
FGF-5	N, H	Mouse	Some	A	+			Haub et al., 1990
EGF	RIA, H	Rat, Mou	Various	D,A	+			Schaudies et al., 1989; Spranger et al., 1990
TGFα	H, I	Rat, Mou	Various	D,A	+	+		Kudlow et al., 1989; Seroogy et al., 1993
HB-EGF	N	Rat	Cx, Hip, MBr	A				Abraham et al., 1993; Vaughan et al., 1992
PDGF-AA	H, I, N	Rat, Mou	All	E,A	+	+		Yeh et al., 1991
PDGF-BB	N, I	Rat	All	E,A	+			Sasahara et al., 1991
IGF-1	H, I, N	Rat, Mou	All	D,A	+	+	+	Lund et al., 1986; Marks et al., 1991
IGF-2	H, N	Rat	All, Chor plexus	D,A				Beck et al., 1988; Haselbacher et al., 1985; Lund et al., 1986; Rotwein et al., 1988
TGFβ1	H, N	Rat, Mou	All, Hip, Cx piri	A		+		Da Cunha and Vitkovic, 1992; Denhez et al., 1990
TGFβ2	I, N	Mouse	Various	D,A	+	+		Denhez et al., 1990; Flanders et al., 1991; Johnson et al., 1993
TGFβ3	I, N	Mouse	Various	D,A	+	+		Denhez et al., 1990; Flanders et al., 1991; Johnson et al., 1993
NGF	H, I, N	Rat	Various	A	+			Korsching et al., 1985; Large et al., 1986
	N	Rat	Opt ner	P12		+		Lu et al., 1991
BDNF	H	Rat	Hip, Cx	A	+			Thoenen, 1991, rev.
NT-3	H	Rat	Hip, Cb	A				Gall et al., 1992; Thoenen, 1991, rev.
CNTF	I, H	Rat	Opt ner, OLB	P8		+		Dobrea et al., 1992; Stöckli et al., 1991
LIF/CDF	N	Rat	Several	D,A				Yamamori, 1991
ET-1	H, I, RIA	Rat, Hu	Sp cord, Cb, Br	A	+	+		Giaid et al., 1991; MacCumber et al., 1990
ET-3	RIA	Rat	Cb, Br	A		+		Matsumoto et al., 1989
Thrombin	N, H	Rat, Hu	Most	D,A	+			Dihanich et al., 1991
GMF	I	Rat	Cb	A	+	+		Lim et al., 1987a; Wang et al., 1992
SCF	H	Rat	Various	D,A				Hirota et al., 1992
IL-1β	N, H	Rat	Some, Hip	A	+	+		Bandtlow et al., 1990; Spranger et al., 1990
IL-6	H	Rat	Various	A		+		Schobitz et al., 1992

Neur, neurons; Astr, astroglial cells; Epen, ependymal cells; N, Northern blot; H, *in situ* hybridization; I, immunocytochemical localization; RIA, radioimmunoassay; Hu, human; Mou, mouse; Chor, choroid; piri, piriform; Sp, spinal; Opt ner, optical nerve; Br, brain; Cb, cerebellum; Cx, cortex; Hip, hippocampus; MBr, midbrain; OLB, olfactory bulb; A, adult; D, development; E, embryo; P, postnatal day. See Tables 30-1 and 30-2 for factors abbreviations.

TABLE 30-4. *Growth Factors Expressed in Astrocytes*

	In Vitro			
Factors	Expr	Inducers	In Vivo Expr	Ref.
aFGF	+		+	Araujo and Cotman, 1992; Ferrara et al., 1988
bFGF	+	IL-1, IL-6, EGF	+	Araujo and Cotman, 1992; Ferrara et al., 1988; Woodward et al., 1992
TGFα	+		+	Kudlow et al., 1989
PDGF-AA	+		+	Dutly and Schwab, 1991; Noble et al., 1988; Yeh et al., 1991
IGF-1	+	Dexamethasone −	+	Adamo et al., 1988; Ballotti et al., 1987; Han et al., 1992
IGF-2	+			Haselbacher et al., 1985; Rotwein et al., 1988
TGFβ1	+	IL-1α, FGF, EGF, TGFβ1	+	Da Cunha and Vitkovic, 1992; Da Cunha et al., 1993; Lindholm et al., 1992; Wesselingh et al., 1990
TGFβ2	+		+	Flanders et al., 1991
TGFβ3	+		+	Constam et al., 1992; Unsicker et al., 1991
NGF	+	Serum, aFGF, bFGF, EGF, PDGF	+	Ladenheim et al., 1993; Rudge et al., 1992; Spranger et al., 1990; Yoshida and Gage, 1991
		TGFα, TGFβ1, ET-1, IL-1, IL-3, IL-6		Zafra et al., 1992
BDNF	±	TPA, forskolin, NE		Rudge et al., 1992; Zafra et al., 1992
NT-3	+			Rudge et al., 1992
CNTF	+		+	Dobrea et al., 1992; Lillien et al., 1988; Rudge et al., 1992; Stöckli et al., 1989
LIF	+	LPS, IFN, TNFα		Wesselingh et al., 1990
GDNF	+			Lin et al., 1993
ET-1	+	ET-1, thrombin	+	Ehrenreich et al., 1991b; Ehrenreich et al., 1993; MacCumber et al., 1990
ET-3	+		+	Ehrenreich et al., 1991a; Matsumoto et al., 1989
GMF	+		+	Lim et al., 1987b
TNFα	0	LPS, IL-1β, substance P, Ca ionophore Newcastle disease virus		Lieberman et al., 1989; Martin et al., 1992; Sawada et al., 1989
IL-1α	0	LPS, TNFα, IL-1β, substance P		Aloisi et al., 1992; Fontana et al., 1982; Lieberman et al., 1989; Martin et al., 1992
IL-1β	0	TNFα, IL-1β	+	Aloisi et al., 1992; Spranger et al., 1990; Tweardy et al., 1990
IL-3	0	LPS	+	Aloisi et al., 1992; Farrar et al., 1989; Ohno et al., 1990
IL-6	0	LPS, IFN, TNFα, IL-1β, NE	+	Aloisi et al., 1992; Schobitz et al., 1992; Wesselingh et al., 1990
IL-8	0	TNFα, IL-1β	+	Aloisi et al., 1992
M-CSF	+	LPS, IFN, TNFα, IL-1β		Aloisi et al., 1992; Wesselingh et al., 1990
G-CSF	0	LPS, IFN, TNFα, IL-1β		Aloisi et al., 1992; Tweardy et al., 1990; Wesselingh et al., 1990
GM-CSF	0	LPS, TNFα, IL-1β		Aloisi et al., 1992; Ohno et al., 1990; Tweardy et al., 1990

Expr, expression; TPA, tissue plasminogen activator; NE, norepinephrine; LPS, lipopolysaccharide. See Tables 30-1 and 30-2 for other abbreviations.

to be high on a large number of specific neuronal populations in the embryonic as well as adult CNS. In contrast, glial cells *in situ* express no or few receptors (Wanaka et al., 1990).

In culture, it has been mentioned that cultured astroglial cells possess FGF receptors (Araujo and Cotman, 1992).

FGFR1, R2, and R3 are all expressed in brain, type-1 on neurons and type-2 on glial cells. Analysis of FGFR1 expression during mouse neurulation has shown high levels in the developing neuroectoderm and as development proceeded, this receptor expression became localized to neuronal precursors. In contrast, FGFR4 mRNA was never detected in the CNS.

The above-mentioned observations suggest that at least aFGF and bFGF and their receptors may be expressed by CNS neurons as well as glial cells.

Effects on Astroblast Proliferation

Fibroblast growth factors and many other growth factors (see also below) have been found to stimulate

the proliferation of quiescent astroblasts (Table 30-5). Acidic and basic FGFs are the most potent with thrombin and EGF. Their effect is transient, and under normal culture conditions they induce only one cell cycle.

Antisense bFGF oligodeoxyribonucleotides inhibit DNA synthesis of rat astrocytes (Gerdes et al., 1992). This result suggests that bFGF, in addition to its classical effect as a growth factor, is also an endogenous growth signal in developing astrocytes.

Effects on Astroblast Morphology and Maturation

FGFs and EGF, elicit a strong morphologic effect on astroblasts (Table 30-5). Their cell bodies shrink, giving rise to small, round cells with extended processes.

Several mitogenic growth factors, including FGFs, have been shown to stimulate the maturation of astroblasts–as determined by their upregulation of the glutamine synthetase (GS) activity and of the level of S-100 protein (Guenter-Lauber and Honegger, 1985; Perraud et al., 1988). These maturation effects follow the transient mitogenic effects of the factors (Loret et al., 1989).

The effects of these growth factors on the rate of synthesis of the various cell proteins have been investigated by using two-dimensional polyacrylamide gel electrophoresis. This method has shown that growth factors are able to modulate the rate of synthesis of many proteins (Table 30-5). This effect lasts for several days after the end of the DNA synthesis and varies with time (Loret et al., 1989).

Effects on O-2A Progenitor Cells and Oligodendrocytes

In subculture and in a chemically defined medium, O-2A progenitor cells and oligodendrocytes proliferate at a very low rate. Two families of growth factors have been found to strongly stimulate their proliferation: FGF and platelet-derived growth factor (PDGF) (Table 30-6). The effect of bFGF can last for several days if the treatment is renewed. Basic FGF also delays the differentiation of O-2A progenitor cells probably as a result of its mitogenic effect (McKinnon et al., 1990).

In reaggregates, bFGF does not elicit a significant mitogenic effect on the O-2A progenitors, but it stimulates cyclic nucleotide phosphohydrolase activity (Honegger and Tenot-Sparti, 1992).

Basic FGF is able to induce the proliferation of 12% of the oligodendrocytes isolated from adult rat brain if the cells were kept for at least 2 weeks *in vitro* (Vick and DeVries, 1992). In mature oligodendrocytes grown *in vitro,* bFGF also inhibits the expression of MBP transcripts (Fressinaud et al., 1995).

Fibroblast Growth Factors and Reactive Gliosis

Reactive gliosis occurs after CNS injury: astrocytes present in and around the area of wound react by a hyperplasia, migration toward the site of wound, and some proliferation. These reactive astrocytes then form a structurally organized posttraumatic glial scar. This subject is treated in Chapter 55, so we will just report some data about the possible involvement of FGF.

In response to a CNS lesion, FGF mRNA and protein levels are increased in macrophages, neurons, astrocytes, and vascular endothelial cells. Reactive astrocytes highly express bFGF within a week and aFGF only after 1 week (Tourbah et al., 1992). A specific expression of the FGFR1 mRNA occurs too.

The injection of bFGF in various brain areas of the neonatal rat after an electrolytic lesion results in an increase of the number of reactive astrocytes as soon as 3 days after injury (Eclancher et al., 1990). After 20 days, the astrocytes are packed in several layers along the borders of the lesion, thus reducing its extension. Similarly, the intraventricular administration of bFGF in adult rat brain after partial fimbria transection enhances the lesion-induced astroglial reaction by changing the morphology of the astrocytes and by increasing the number of the reactive astrocytes that proliferate. It is suggested that bFGF-promoted glial scar could be beneficial for wound repair and neural sprouting in the damaged brain.

EPIDERMAL GROWTH FACTOR

Family

The epidermal growth factor (EGF) family is composed of at least five factors that bind to the same receptor (Table 30-1). TGFα has been discovered (like TGFβ) as a molecule secreted by fibroblasts transformed by a retrovirus. EGF, TGFα, HB-EGF, and EGFR are present in brain (Table 30-3). TGFα is localized mainly in neurons but is also found in a subpopulation of astrocytes (Kudlow et al., 1989). The receptor is expressed in astrocytes and in ependymal cells (Birecree et al., 1991).

TABLE 30-5. *Effects of Growth Factors on Astrocytes*

Factors	Species	Cells origin	Effects				Ref.
			Pro	Mor	Mat	Other effects ±	
a or bFGF	Rat	Hem	+	+	+	Modulation of protein synthesis	Loret et al., 1989; Perraud et al., 1988; Pruss et al., 1982
	Rat	Brain				NGF production +	Yoshida and Gage, 1991
	Rat	Hem				IGFBPs, IGF-1 release +	Pons and Torres, 1992
EGF	Hu, Rat	787CG,Cb	+	+		Modulation of protein synthesis	Leutz and Schachner, 1981; Westermark, 1976
	Rat	Hem	+	+	+	Modulation of protein synthesis	Loret et al., 1989
SDGF	Rat	Hem	+				Kimura et al., 1990
PDGF-AA	Rat	Hem	+	+			Giacobini et al., 1992
PDGF-BB	Hu	787CG	+				Heldin et al., 1977
	Rat	Hem	+	+	+	Chemotactic	Besnard et al., 1987; Bressler et al., 1985
IGF-1	Rat	Cortex	+			bFGF, EGF mitogenic effects +	Han et al., 1987; Han et al., 1992
IGF-2	Rat	Cortex	+				Han et al., 1987
TGFβ1	Rat	Brainstem	+	+		MHC-II, IFN induced −	Johns et al., 1992
	Rat	Hem	−		−	Mitogenic effect of FGF +	Labourdette et al., 1990
TGFβ2	Rat	Hem	−		−		Chao et al., 1992; Johnson et al., 1993
TGFβ3	Rat	Hem	−				Johnson et al., 1993
ET-1	Rat	Cb	+			Intracellular Ca^+, K^+ channels +	Suppatapone et al., 1989
	Mouse	Hem				Permeable junctions −	Giaume et al., 1992
ET-2	Rat	Hem				PIP turnover +	Lin et al., 1990
ET-3	Rat	Hem				cAMP morphological effect −	Koyama et al., 1993
Thrombin	Rat	Hem	+		+	Modulation of protein synthesis	Loret et al., 1989; Perraud et al., 1987
	Rat	Hem				cAMP-induced morphology −	Cavanaugh et al., 1990
GMF	Rat	Hem	+	+	+		Kato et al., 1981
uPA	Rat	Cb	+				Moonen et al., 1985
PD-ECGF	Rat	Brain	−				Asai et al., 1992a
VIP	Rat	Sp. cord	+			Secretion of neuron survival factors +	Brennenman et al., 1990
TNFα	Hu	Hem	+			VLA-1, 2, 6, MHC-I +	Barna et al., 1990; Hurwitz et al., 1992
IL-1β	Hu, Rat	Hem				TNFα, IL-6, NGF expression +	Gadient et al., 1990
IL-1	Rat	Hem	+			PA inhibitor, PGE2 +	Giulian and Lachman, 1985
	Hu	Hem				VLA-1, -2, -6 +	Rogister et al., 1990
BCGF	Rat	Hem	+	+	+		Benveniste et al., 1989
IFNγ	Hu, Mou.	Cortex	+			VLA-1, -2, -6, MHC-I, -II +	Hirsch et al., 1983; Hurwitz et al., 1992; Selmaj et al., 1990

Hem, brain hemispheres; Cb, cerebellum; Pro, proliferation; Mor, morphology; Mat, maturation; +, positive effect; −, negative effect; VLA, vascular adhesion molecule; uPA, urokinase-type plasminogen activator; Hu, human; Sp, spinal; MHC, major histocompatibility complex; PIP, phosphoinositol phosphate; BCGF, B cell growth factor. See Tables 30-1 and 30-2 for other abbreviations.

Effects on Glia

Westermark (1976) first reported that EGF was able to stimulate the proliferation of the human glial cell line 787CG. EGF also stimulates the proliferation of astroblasts in primary culture (Table 30-5). It induces a morphological transformation of these cells, stimulates the activity of GS, and modulates the rate of synthesis of many proteins (Loret et al., 1989).

A strong correlation has been found between tumor grade of glioblastomas and the extent of TGFα expression. High expression and structural abnormalities of the EGF receptor have been found to provide tumorigenic advantage for gliomas.

EGF has been reported to inhibit myelin basic protein expression in mature oligodendrocytes (Sheng et al., 1989) (Table 30-6).

PLATELET-DERIVED GROWTH FACTORS

Three platelet-derived growth factors (PDGFs) are known as combinations of two peptidic chains: AA, AB, and BB (Table 30-1). They have been discovered in the blood platelets from which they are released upon activation. These factors are found in various normal and transformed cells and stimulate mainly the growth of mesodermal cells. They are present in brain, mainly in neurons, but PDGF-AA mRNA is also weakly expressed in astrocytes *in vivo* (Table 30-3). *In vitro*, this factor is expressed and secreted by astrocytes (Table 30-4) and by oligodendrocytes.

Effects on Astroblasts

Under the effect of PDGFs, astroblasts in culture are induced to proliferate, they extend processes, but

TABLE 30-6. *Effects of Growth Factors on the Oligodendroglial Cells*

		Effects on O-2A Progenitors				Effects on Oligodendrocytes			
			Differ into						
Factors	Species	Pro	OL	A2	Other	Pro	Matur	Other	Ref.
aFGF	Rat	+	−	−					Besnard et al., 1989b
bFGF	Rat	+	−	−		+			Besnard et al., 1989b; Eccleston and Silberberg, 1985
	Rat	+	−	−	PDGFR +				McKinnon et al., 1990
a or bFGF	Rat					+			Vick and DeVries, 1992
EGF	Mouse	0					MBP −		Sheng et al., 1989
PDGF-AA	Rat	+	−	−				Fos, Jun +	Hart et al., 1992; Richardson et al., 1988
PDGF-BB	Rat	+	−	−					Besnard et al., 1987
IGF-1	Rat	+	+				CNP +		McMorris et al., 1986; Van der Pal et al., 1988
	Rat		+						McMorris and Dubois-Dalcq, 1988
TGFβ1	Rat	+			FGF Pro −				Besnard et al., 1989a
NGF	Pig	0	0	0		+			Althaus et al., 1992
CNTF	Rat	0		+				Survival +	Hughes et al., 1988; Lillien et al., 1988; Louis et al., 1993
TNFα	Mouse							Death	Selmaj and Raine, 1988
IL-2	Rat	+					PLP −		Benveniste and Merril, 1986; Sessa et al., 1992

Pro, proliferation; Differ, differentiation; OL, oligodendrocytes; A2, type-2 astrocytes; Matur, maturation; +, positive effect; −, negative effect; 0, no effect. See Tables 30-1 and 30-2 for other abbreviations.

much shorter than under the effect of FGF, and they increase their synthesis of thrombospondin, a 450 kD lectin-like protein. This factor elicits also a chemotactic effect on astroblasts *in vitro* (Table 30-5).

Trapidil, which is known as a specific antagonist of PDGF, is able to reduce gliosis, a result suggesting that PDGF is involved in this reaction.

Effects on O-2A Progenitors and Oligodendrocytes

PDGFs are mitogens for O-2A progenitor cells in monolayer cultures, but their effect is more transient than that of FGFs. PDGF-AA is more active than PDGF-BB on the growth of optic nerve O-2A progenitors. Like FGFs, PDGFs delay the differentiation of the progenitors (Table 30-6). The combination of FGF and PDGF is more efficient to maintain a high rate of proliferation and to inhibit O-2A differentiation (Bögler et al., 1990).

The mitogenic effect of astroblast-conditioned medium on O-2A progenitors appears to be due to PDGF (Noble et al., 1988). In mixed cultures of astroblasts and O-2A progenitor cells, the fast proliferation of O-2A progenitor cells and their low rate of differentiation may be due to the presence of serum in the culture medium but also to PDGF synthesized by type-1 astroblasts.

PDGF is not a mitogen for mature oligodendrocytes but it induces the expression of Fos and Jun proteins in these cells, suggesting an activation of the receptors. In reaggregates, PDGFs are weak mitogens for glia (unspecified) and they stimulate CNP (Honegger and Tenot-Sparti, 1992).

INSULIN-LIKE GROWTH FACTORS

The insulin-like growth factor family is composed of IGF-1, IGF-2, and also insulin, which is known as a hormone. These three factors are found mainly in serum. The two IGFs are produced at a high level by liver. IGF-1 and its receptor are synthesized in most tissues and cells, and in the brain in which a truncated form is found. IGF-2 mRNA was also found in brain, in choroid plexus, meninges, and vascular sheaths (Table 30-3). Its presence in astrocytes and ependymal cells is still controversial. *In vitro*, astrocytes synthesize IGF-1 and IGF-2 (Table 30-4). Insulin is present in brain, but its origin is not known (Wozniak et al., 1993).

The presence of IGF-1 or of insulin at a high level is required to keep the various glial cells surviving *in vitro*. However, the cells can survive for a few days without insulin. Under this condition, the mitogenic effects of the other growth factors are much lower. Moreover, the addition of anti-IGF-1 antibody to the culture medium prevents the mitogenic effect of EGF on astrocytes (Chernausek, 1993). Thus IGF-1 appears to be a cofactor of the mitogenic effects of the growth factors. Alone, IGF-1 is a weak

mitogen for astrocytes and oligodendrocyte O-2A progenitors, and promotes the differentiation of the latter cells into oligodendrocytes (Table 30-6). In oligodendroglial cells, it increases the level of expression of MBP, CNP, and glycerol-phosphate dehydrogenase (G3PDH) (van der Pal et al., 1988). A general effect of IGF-1 on oligodendrocytes is shown in transgenic mice overexpressing IGF-1. In these animals, the percentage of oligodendrocytes in brain is not modified, but the amount of myelin is increased by 130% (Carson et al., 1993).

C6 glioma cells expressing IGF-1 antisense RNA lose their tumorigenicity and prevent that of non-transfected C6 cells. This observation suggests that antisense blocking of IGF-1 expression may reverse a phenotype that allows C6 cells to evade the immune system (Trojan et al., 1993).

TRANSFORMING GROWTH FACTORS TYPE β

Family

TGFβ1 has been discovered as a molecule secreted with TGFα by fibroblasts transformed by retroviruses. It has the same primary structure in man, pig, bovine, and monkey, but in murine there is one different amino acid. This factor inhibits the growth of most cells, but it can be a mitogen for some other cells. Most, if not all, cells have receptors for TGFβs.

TGFβs and transcripts of the three isoforms (Table 30-1) are expressed in embryonic and adult mouse brain (Table 30-3), but the presence of TGFβ1 in neural cells is still debated. Astrocytes express the three forms *in vivo* as well as *in vitro* (Table 30-4).

Effects on Astroblasts

TGFβ1 inhibits the proliferation of astroblasts, but a stimulatory effect has also been reported (Table 30-5). It stimulates phosphoinositol metabolism and the translocation of protein kinase C (Robertson et al., 1988). In these cells, it potentiates the mitogenic effect of bFGF but not that of the other growth factors, and it inhibits transiently the morphologic effect of bFGF (Table 30-5).

Effects on O-2A Progenitor Cells

Alone, TGFβ1 stimulates weakly the proliferation of O-2A progenitor cells (Table 30-6). When it is combined with bFGF, its effect is bimodal. At low concentrations it potentiates the mitogenic effect of bFGF, and at higher concentrations it inhibits it (Besnard et al., 1989a).

NEUROTROPHIC FACTORS

Neurotrophic factors have been defined primarily as molecules involved in the regulation of a variety of neuronal developmental processes such as differentiation, survival, neurite outgrowth, and synaptic rearrangement.

In the adult CNS, glial cells do not express, or at relatively low levels, neurotrophic factors (such as CNTF and NGF) under normal conditions (Table 30-3). However, following injury the synthesis of such factors increases dramatically and transiently in the area around the wound site and become mainly localized in reactive astrocytes forming the glial scar. Growing astroglial cells in culture results in sustained expression of neurotrophic factors. Thus, astrocytes *in vitro* may behave just as the activated astrocytes *in vivo* by upregulating their levels of neurotrophic factors.

Neurotrophins and Their Receptors

As reported above, NGF, a basic 118-amino acid protein, was the first growth factor (actually a neurotrophic factor) discovered. Subsequently, several neurotrophic factors belonging to the family of NGF, called neurotrophins or neurokines, were characterized. These factors, brain-derived neurotrophic factor (BDNF), NT-3, NT-4, and NT-5, have a strong sequence homology, but they exhibit different neuronal specificity. All five neurotrophins are capable of promoting the survival and differentiation of sensory neurons of the peripheral nervous system. NGF is also a survival factor for sympathetic neurons. BDNF supports in addition retina ganglion cells, mesencephalic dopaminergic neurons and neural crest-derived sensory neurons. BDNF as well as NT-3 affect neurons of the nodose ganglion. Cholinergic neurons in the basal forebrain respond to both NGF and BDNF (for reviews, see Thoenen, 1991; Ebendal, 1992; see also Chapter 29, this volume). Recently, a new neurotrophic factor, GDNF (glial cell line-derived neurotrophic factor) has been characterized. It is active on midbrain dopaminergic neurons (Lin et al., 1993).

The first known NGF receptor was a low-affinity receptor (named $p75^{NGFR}$ or LNGFR, low-affinity nerve growth factor receptor or LANR, low-affinity neurotrophin receptor) acting probably through interaction with G proteins (Figure 30-1). Subsequently, the protooncogene *trk* (*trk*A), named

because of its tyrosine kinase activity, has been shown to be a high-affinity receptor for NGF. Later on, two other high-affinity receptors related to *trk* (*trk*B and *trk*C) have been cloned and characterized. LNGFR binds all neurotrophins while *trk*A binds NGF and NT-5, *trk*B binds BDNF and NT-3 to -5, and *trk*C binds NT-3. It seems that the low- and high-affinity receptors can act independently, triggering different biological effects; however, not all data fit with this view (for review see Meakin and Shooter, 1992).

The *trk*B receptors expressed by glial cells *in vivo* as well as *in vitro,* are truncated forms, lacking the tyrosine kinase domain (Frisén et al., 1993). The high expression of these truncated *trk*B receptors by glial cells in the intact nervous system and their altered expression after injury, as shown by an increase or decrease in the injured CNS or peripheral nervous system, respectively point out to a functionally important involvement of this nonneuronal NGF receptor in nerve regeneration.

The truncated forms of *trk*B and *trk*C, notably expressed in astrocytes, may act not as signaling receptors, but rather as neurotrophin-trapping molecules (Valenzuela et al., 1993).

In Vivo **Expression.** The members of the neurotrophin family show parallel as well as distinct patterns of regional expression in the CNS. The hippocampus and neocortex are the brain regions with the highest levels of NGF and BDNF. The largest amounts of NT-3 are found in the hippocampus and cerebellum (Table 30-3). These three neurotrophic factors are maximally expressed at different times of brain development in the rat (for review see Thoenen, 1991).

Nerve growth factor protein has been found to be predominantly localized in neurons of various brain areas. However, actively growing glial cells in the developing or injured CNS may as well synthesize NGF (Lu et al., 1991). Transection of the adult optic nerve, which is known to elicit increase in reactive glial cell number, evokes a marked and transient increase in NGF mRNA expression. Another study has demonstrated that after lesion of the septohippocampal pathways or by intraventricular administration of IL-1β, many astrocytes activated by both treatments become NGF-like immunoreactive in the septum and hippocampus (Oberfeld-Nowak et al., 1992). This expression does not appear to be related to proliferation.

In the CNS of the rat, NGF receptor mRNA and protein have been detected mainly in neurons during development. However, in the adult rat brain, NGF receptors have also been detected in ependymal cells and in nonnervous cells such as leptomeninges, epithelial cells of the plexus choroid and in the cerebral vessels (Delvalle et al., 1992). Another study (Frisen et al., 1992a) has shown that *trk*B mRNA as well as *trk*B protein-like immunoreactivity are increased in the rat and cat spinal cord after trauma, suggesting a role for these receptors in axonal sprouting. The *trk*B receptors have been localized to neurons, but also to astrocytes and leptomeningeal cells. However, as mentioned above, the *trk*B and *trk*C receptors expressed in glia are inactive forms.

In Vitro **Expression.** Cultured neuronal and glial cells (specifically astrocytes, Table 30-4) from a variety of brain areas express the mRNA for NGF and synthesize and secrete the protein (Zafra et al., 1992).

In culture, rat hypothalamic and cortical oligodendrocytes, a subpopulation of differentiated astrocytes, and a subpopulation of hypothalamic neurons express the NGF protein but at different levels (Gonzales et al., 1990) while NGF mRNA is expressed at similar levels by cortical type-1 astrocytes, oligodendrocyte progenitor cells and neurons. This observation suggests the existence of different posttranscriptional regulations in these cell types. In other culture systems, rat brain oligodendrocytes and microglial cells do not express NGF mRNA, and the message for NGF has only been detected in astrocytes (Spranger et al., 1990).

In low-density cultures, in which glial cells grow rapidly, the cells express high levels of NGF mRNA and as density increases with time, amounts of NGF mRNA decrease progressively. Thus, actively growing glia in culture may preferentially synthesize NGF.

Cultured rat hippocampal astrocytes have been found to express mRNA for NGF and NT-3 at significantly higher levels than cultured neuronal cells. However, NGF and NT-3 proteins are undetectable immunocytochemically. In contrast, these astrocytes do not express BDNF mRNA, but the message is detectable in the cultured hippocampal neurons (Rudge et al., 1992; Zafra et al., 1992).

The production of NGF by astrocytes in culture is extremely variable and can be regulated by many molecules (Table 30-4). The lymphokine interleukin-1 (IL-1) and several growth factors, such as EGF, aFGF, bFGF, TGFα, and TGFβ stimulate transiently the synthesis of NGF by cultured astrocytes (Spranger et al., 1990). The increase of NGF mRNA is reflected by a corresponding increase in NGF protein release into the culture medium (Spranger et al., 1990; Yoshida and Gage, 1991). It has been described that cytokines, such as IL-1 and TGFβ1 are without effect on BDNF and NGF mRNA levels in cultured neurons (Zafra et al., 1992). These authors also reported that all the cytokines and growth factors that produce a marked increase of NGF mRNA

in cultured astroglial cells are without effect on astrocyte BDNF mRNA levels. Thus, it seems that there are notable differences in the regulation of BDNF and NGF mRNA expression in cultured astrocytes.

Effects on Glia. Human fetal astrocytes do not express functional high-affinity NGF receptors (see the section *Neurotrophins and their Receptors* above) and do not respond to NGF. In contrast, NGF has been reported to induce proliferation and enhances fiber regeneration in mature oligodendrocytes from adult pig brain (Table 30-6).

Ciliary Neurotrophic Factor, Cholinergic Differentiation Factor/Leukemia Inhibitory Factor, and Interleukin-6

CNTF, CDF/LIF, and IL-6 (together with oncostatin M) are structurally related molecules which elicit overlapping effects on various cells. This characteristic appears to be due to the structure of their receptors (Figure 30-2), which are complexes sharing the gp130 tyrosine kinase. It seems that gp130 alone has no binding capacity for any of the three factors.

CNTF, a 20 to 24 kD acidic protein, has been first found and isolated from chick embryonic ocular tissue. This factor has been shown to support *in vitro* survival of parasympathetic ciliary ganglion neurons and of a variety of other peripheral neurons as well as CNS neurons. CDF/LIF has been purified by several groups under various names, since it elicits effects on cells in many organs. In the nervous system, this factor induces the cholinergic differentiation of mature sympathetic neurons. More details about IL-6 are given in the section *Cytokines and Hematopoietic Factors* below (seee also Chapter 46, this volume).

***In Vivo* and *In Vitro* Expression.** In the adult rat CNS, CNTF mRNA and protein levels are high in the olfactory bulb and optic nerve but barely detectable in the rest of the brain (Stöckli et al., 1991). CNTF immunoreactivity is restricted to a subpopulation of cells with an astrocyte-like morphology (Table 30-3). IL-6 is also localized in astrocytes *in vivo*. CDF/LIF is present in various areas of the brain, but its cellular localization has not yet been determined.

Astrocytes from newborn rat brain or hippocampus in culture express high levels of CNTF mRNA and protein. Expression of CDF/LIF has also been observed in astrocytes *in vitro*, and it is stimulated by other cytokines and LPS (Table 30-4).

The CNTF protein does not contain a secretion signal sequence, indicating that this molecule is not released, or not by the usual mechanism. However, in the denervated nerve, CNTF was found to be localized extracellularly and may play an important role during neurite outgrowth (Sendtner et al., 1992; see also Chapter 29, this volume).

Effects on O-2A Progenitors and Oligodendrocytes. *In vitro*, CNTF induces bipotential glial O-2A progenitor cells of rat optic nerve and brain to differentiate into type-2 astrocytes, and more efficiently in cooperation with extracellular matrix-associated molecules (Lillien et al., 1990). CNTF also protects oligodendrocytes against death *in vitro* (Table 30-6).

ENDOTHELINS

Endothelin (ET) was first characterized as a vasoconstrictor peptide produced by endothelial cells (see also Chapter 24). It stimulates DNA synthesis in vascular smooth muscle cells. Three highly homologous peptides have been identified.

Endothelins and binding sites are found in brain, in neurons, and in glial cells (Table 30-3). *In vitro*, astrocytes produce ET-1 and ET-3 (Table 30-5) and their receptors (Ehrenreich et al., 1991a). Endothelins elicit various effects on astrocytes including proliferation (Table 30-5). Of particular interest is their stimulatory effect on NGF (Table 30-4) and c-*fos* expression (Ladenheim et al., 1993).

THROMBIN

The thrombin molecule was first known as a protease, secreted by blood platelets and acting on the blood clotting. It is also a growth factor with a mitogenic activity toward fibroblasts, macrophages and endothelial cells. Its receptor has been recently characterized and cloned. Thrombin and its receptor are expressed in brain and only in neurons (Table 30-3).

Thrombin is a mitogen for astroblasts (Table 30-5) and stimulates S6 kinase through a protein kinase C-dependent mechanism and stimulates the activity of GS (Loret et al., 1989). In contrast to the effects of FGF and EGF which make the cells fibrous, after treatment with thrombin astroblasts are flatter than without treatment. Thrombin also reverses the stellation induced by dibutyryl cyclic AMP (Table 30-5).

GLIA MATURATION FACTOR

The glia maturation factor (GMF), a 17 kD acidic protein, has been first purified from pig brain. It stimulates the morphological and chemical matura-

tion and the proliferation of rat astroglial cells in culture (Table 30-5).

GMF is synthesized mainly in the nervous system. By using a monoclonal antibody, GMF has been localized in rat spinal cord astrocytes and rat cerebellum Bergman glia (Lim et al., 1987a). The presence of GMF in glial cells has been confirmed by a study on cultured rat brain astroglial cells (Lim et al., 1987b). In contrast, a polyclonal antibody developed against recombinant human GMF has been found to stain astrocytes and neurons as well (Table 30-3). Thus, GMF could mediate communication among astrocytes, among neurons, and/or between neurons and astrocytes.

PLATELET-DERIVED ENDOTHELIAL CELL GROWTH FACTOR OR GLIOSTATIN

Gliostatin, a polypeptide of two 50 kD chains, inhibits the growth of astrocytes *in vitro* (Table 30-5). Further work has shown that gliostatin is identical to PD-ECGF (Asai et al., 1992b).

CYTOKINES AND HEMATOPOIETIC FACTORS

Section IX of this volume is devoted to glia as part of the immune system so we will give only a few data in this field.

Interleukins are polypeptide factors acting as intermediates between leukocytes, but some of them like IL-1, IL-3, and IL-6 are also involved in other systems and particularly in the nervous system in which their functions seem to be specific.

IL-1 is synthesized by various cells and is responsible for the reactions of the organism to inflammation.

TNFα has been isolated from the serum of mouse treated with LPS (bacterial lipopolysaccharides) on the basis of its necrotic action on mouse sarcomas. This immunoregulator is produced mainly by lymphocytes and macrophages. *In vitro,* TNF-α is in most cases an inhibitor of cell growth. All cells, with the exception of red blood cells, express TNF-α receptors.

Colony stimulating factors (CSF) are mainly involved in the control of the development of hematopoietic cells.

Astrocytes and Cytokines

Astrocytes are immunocompetent cells that could participate locally to immunological reactions. In mouse brain infected with trypanosoma, the astrocytes express GM-CSF, TNFα, IL-6, IFNγ, and higher levels of IL-1α (Hunter et al., 1992).

In vitro, IL-1 and TNFα stimulate the proliferation of astrocytes and elicit various effects on these cells (Tables 30-4 and 30-5).

Astrocytes activated by endotoxins, viruses, and the cytokines IL-1, TNFα, and IFNs, produce several interleukins, the three CSFs, and on occasion TNFα and IFNs (Table 30-4). The expression of MHC-II is induced by TNFα and IFNγ. M-CSF is expressed in normal cultured astrocytes. TNFα, IL-1, and LPS stimulate its expression through a posttranscriptional regulation (Frei et al., 1992).

TNFα induces the expression of several adhesion molecules and of their mRNAs in cultured human fetal astrocytes (Table 30-5). Thus, in inflammatory conditions, the expression of these molecules by the astrocytes could facilitate the migration of leukocytes and contribute to the development of diseases like multiple sclerosis (Hurwitz et al., 1992).

Many astrocytoma and glioblastoma cells express and release IL-6 *in vitro* and *in vivo.*

High concentration of IL-2 stimulates the proliferation of O-2A progenitor cells (Table 30-6). IL-1 increases the number of IL-2 receptors in these cells. TNFα induces the death of oligodendrocytes *in vitro.*

B Cell Growth Factor

B cell growth factor is a T cell-derived lymphokine. It induces the growth of rat astrocytes, elicits a morphological effect on these cells, and enhances the level of GFAP mRNA (Table 30-5).

PUTATIVE FUNCTIONS OF GROWTH FACTORS IN THE CENTRAL NERVOUS SYSTEM

In a pluricellular organism, the behavior of every cell is governed exclusively by interactive processes between the signals it receives from the outside and its genetic program. Such interplay will differ somehow in the three different physiological conditions a cell will face: development, adulthood, and trauma. Growth factors are probably the main signals involved in these interactions, in addition to other soluble or insoluble molecules, and to direct intercellular contacts. The expression and the reception of some of these other signals and intercellular contact molecules can also be regulated by growth factors. The variety of the growth factors, their receptors, their localization, their direct and indirect effects on the cells, and the timing of their expressions in the

central nervous system can generate a tremendous number of combinations in space and in time. This number of possibilities is compatible with the highly specific and punctual actions required for the formation and maintenance of a complicated structure like the brain.

Involvement of growth factors in the development of the central nervous system is suggested by variations of their expressions and their receptors with time. Such an involvement is probable, as seen by the effects of growth factors on developmental processes like proliferation, differentiation, and morphological effects on nerve cells in culture. It is directly shown, for instance, by the greater development of brain in IGF-1 transgenic mice, compared to normal animals.

Involvement of growth factors in the adult brain is suggested also by the presence of various growth factors and by various observations concerning the maintenance, functioning, and plasticity of the brain. For example, as reported before, an increase of NGF concentration is found in hippocampus of male mice after fighting. Also, an increase in aFGF production and release by ependymal cells follows food intake by mice and that increase participates in the induction of the subsequent feeding avoidance.

Involvement of growth factors in response to trauma is likely, considering all the data available on that subject. After injury, several growth factors that are not produced in the normal brain are specifically synthesized and released at the site of injury and around it, and some of them are involved in the immunological defense. Intracerebral injections of some growth factors can improve neuronal survival or can mimic or enhance reactive gliosis. On the other hand, intracerebral injections of growth factor antagonists or antibodies can reduce or modify glial responses.

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31 Effects of nerve impulses on glial membranes

RICHARD K. ORKAND

Coordination of glial cell function with neuronal activity requires mechanisms whereby neurons signal their behavior to the glial cells and affect their activity. The idea of neuron-glia interactions can be traced at least to Ramón y Cajal in 1895, who proposed a role for glial cells in higher nervous system function, based on his observations suggesting glial motility:

> The unpredictable turns that associations sometimes take—the fading away of ideas and words, the momentary halting of speech, the obsessive persistence of memory, the repression of an idea or experience as well as all types of erroneous motor reactions and other psychological function can be understood . . . by supposing the neuroglia of the gray matter serve as an insulating switching mechanism for nervous currents, permitting connections when they are active, and acting as insulators during repose (Ramón y Cajal, 1895, cited by Galambos, 1971).

Over longer times, such as those that are important during development (Chapters 27 and 49), myelination (Chapter 40), regeneration (Section XI), degeneration, and the activation of early response genes (Chapter 35), such signals, although not always well defined, must exist; they are discussed elsewhere in this volume. The possibility of relatively rapid effects of neurons on glial cells, which are important to glial function, becomes stronger as data accumulate, indicating that glial cells possess receptors for a number of small molecules which behave as transmitters or modulators at conventional synapses (see Section IV). Moreover, as it becomes clear that these potential messengers may be released from glial cells under appropriate conditions, the possibility of rapid signals from glial cells to axons must also be considered. It is well established that glial cells communicate with each other via gap junctions, which permit the exchange of ions and small molecules (Chapters 17 and 19).

This chapter deals mainly with possible interactions resulting from nerve impulse activity that are effective during shorter periods (seconds and minutes) and that might provide a signal for glial cells to function in the homeostasis of the neuronal microenvironment with regard to ions and small molecules. Among these homeostatic functions are the roles of glial cells in potassium regulation (Chapters 18 and 47), transmitter removal (Chapter 48), release of substrates (Chapter 52), and the buffering of extracellular pH (Chapter 14).

ANATOMICAL RELATIONS

Usually the membranes of glial cells and neurons are separated by narrow extracellular clefts, of the order of 15 nm wide, which form an intersecting network of sheetlike spaces (Chapter 26). This fluid space permits the diffusion of ions and small molecules between and among the two cell types. Thus, a substance released from one cell may readily affect another, depending on the diffusion coefficient for the particular substance in the space, the ability of the cells to modify the concentration of the substance through uptake or metabolism, the availability of receptors, and the resulting concentration of the substance at the receptor sites (Nicholson and Rice, 1991). Therefore, to consider whether a substance released from neurons has an effect on glial cells we need to consider the amount of the substance released from the neuron, the fate of the substance in the extracellular space, the availability of receptors on the glial cells, and the concentration of the substance reaching the glial receptors. Figure 31-1 depicts the anatomical relations of the cells of the central nervous system of vertebrates.

Special Cases

The anatomical relations between glial cells and neurons are highly specialized in the case of the ensheathement of axons by oligodendrocytes to form myelin (Chapter 40). At the nodes of Ranvier the terminal loops formed by oligodendrocyte membrane come in close contact with the axon and form an especially

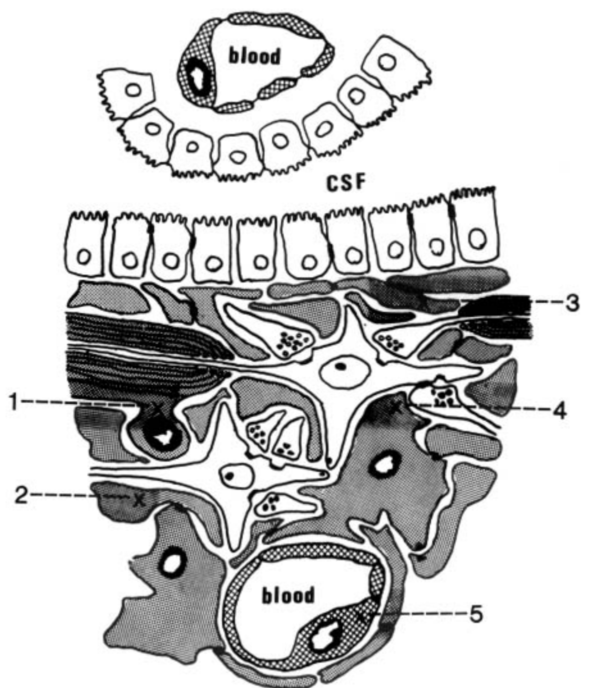

FIG. 31-1. Some cellular relations in the vertebrate nervous system. (**Top**) Blood capillary perfusing choroid epithelium. Below the capillary are choroid epithelial cells. These cells, joined by tight junctions, secrete cerebrospinal fluid (CSF). Below the CSF are ependymal cells joined only by spot attachments at gap junctions. This allows free diffusion between the CSF and the intercellular clefts. Glial cells (*shaded areas*) are within the ependyma. The glial cells are joined by gap junctions. Presynaptic terminals contain synaptic vesicles. An oligodendrocyte (*1*) enwraps an axon to produce myelin. The major sites where astrocytes are exposed to substances released from neurons are along unmyelinated axons (*2*), at breaks in the myelin (*3*), and in the region of synapses (*4*). (**Bottom**) A brain capillary is surrounded by astrocyte endfeet with occasional gap junctions permitting diffusion from the extravascular space to the intercellular clefts. The endothelial cells (*5*) forming the capillary are joined by tight junctions and constitute the blood-brain barrier. [Modified from R. Orkand (1969), with permission.]

close apposition (cf. Chapter 51). Moreover, in a number of fiber tracts, there is a special anatomical relation between astrocyte processes and the node which makes the astrocyte part of a "paranodal apparatus" (Black and Waxman, 1988) and suggests a special functional relation at that site. Immunolocalization studies have demonstrated Na^{+} channels in these perinodal astrocytes (Black et al., 1991). However, the functional implications of this finding are not yet clear.

In at least one specific region of the nervous system, the pituitary pars intermedia, neurons form actual synaptic contacts with stellate cells, which have been identified as being glial because they stain positively for glial fibrillary acidic protein (GFAP). A calcium-dependent postsynaptic response consisting of a short (100 ms) depolarization followed by a more prolonged (45 to 75 s) hyperpolarization has been recorded from these glial cells (Mudrick-Donnon et al., 1993). The depolarization is mimicked by gamma-aminobutyric acid (GABA) and the hyperpolarization by dopamine. These experiments provide the first evidence for synaptic potentials in glial cells, and the results are consistent with this unusual anatomical arrangement.

NERVE IMPULSES DO NOT DIRECTLY AFFECT GLIAL CELLS

In the absence of such special anatomical relations, the question arises as to whether the flow of ionic current in the neurons might by itself exert an effect on the glial cells. There is no evidence that it does so. Kuffler and Potter (1964) recorded the membrane potential of packet glial cells in the leech while changing the membrane potential in the adjacent Retzius nerve cells. Neither massive neuronal discharge or prolonged potential changes produced by passing current in the neuron produced significant membrane potential changes across the glial membrane. R. Orkand et al. (1966) recorded the action potential with a microelectrode in the extracellular space of a mudpuppy optic nerve during a maximal nerve volley, advanced the electrode into a glial cell and again stimulated the axons. As shown in Figure 31-2, the action potential recorded by a microelectrode had the same form and amplitude when recorded with the electrode in the cell as it did when outside. This indicates that the recorded potential is the result of current flow in the extracellular space across the resistance of the extracellular fluid and does not produce a significant potential change across the glial membrane. The results can readily be explained by supposing that the extracellular space between the neurons and glia shunts the neuronal currents, that is, that the effective resistance of the glial membrane is much larger than that of the extracellular clefts.

NERVE IMPULSES ALTER THE GLIAL MICROENVIRONMENT

Ionic Changes Resulting from Impulse Activity

Potassium. The suggestion that nerve impulses cause changes in the ionic composition of the extracellular fluid and that these changes can have functional consequences was put forward by Barron and Matthews (1938) to explain negative dorsal root potentials and some other extracellular potentials recorded from

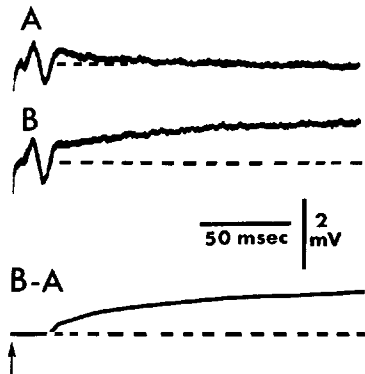

FIG. 31-2. Lack of effect of external current generated by nerve impulses on the glial membrane potential. (**A**) A recording microelectrode was placed in the extracellular space of an optic nerve of *Necturus* ($V_m = 0$) and the nerve was electrically stimulated. The triphasic potential is caused by current flow from the active axons across the resistance in the extracellular fluid between the microelectrode and the indifferent electrode in the bathing solution. (**B**) The electrode was advanced slightly and recorded a glial resting potential ($V_m = -84$ mV). Identical nerve stimulation sets up the same triphasic potential which, however, is followed by a slowly rising glial depolarization. *B − A*: Graphic subtraction of the two traces shows that the triphasic current from the stimulated axons did not produce a potential across the glial membrane. The remaining slow depolarization is the result of K^+ accumulation in the extracellular space following its efflux from the active axons primarily during the falling phase of the action potential. [Modified from R. Orkand et al. (1966), with permission.]

the spinal cord. In a study of afterpotentials in the squid giant axon, Frankenheuser and Hodgkin (1956) concluded that repetitive stimulation of the axon led to an accumulation of K^+ in the periaxonal space and that this change in the electrochemical driving force for K^+ (E_K) accounted quantitatively for the progressive reduction in hyperpolarizing afterpotentials and depolarization of the axon (Baylor and Nicholls, 1969; Astion et al., 1988). A similar mechanism was later suggested to explain the depolarization of glial cells in the amphibian optic nerve following axon stimulation (R. Orkand et al., 1966; see section—*Nerve Impulses Are Detected By Glial Cells*) and Figure 31-3). The development of ion-selective electrodes, especially those to measure K^+ in the extracellular space of the nervous system, has permitted extensive studies of the changes in this ion during normal and pathological nervous activity. These studies have been reviewed in this volume (see Chapters 26 and 47) and elsewhere (Sykova, 1983, 1992).

Sodium and Chloride. Sodium and chloride are the most abundant ions in the extracellular fluid and the movement of these ions across nerve membranes during activity does not produce large fractional changes in their extracellular concentration, and therefore they have little effect on their equilibrium potentials. Whereas a change of a few millimoles in extracellular K^+ produces relatively large membrane potential changes in glia (Kuffler et al., 1966; Kettenmann et al., 1983) and could affect glial activity, comparable changes in Na^+ or Cl^- concentrations do not have the same effect (Coles et al., 1989). However, during pathological situations, like spreading depression (Kraig and Nicholson, 1978), dramatic changes in Na^+ and Cl^- might have functional consequences for the glial cells.

Calcium. Calcium activity in the extracellular fluid is low ($1–5 \times 10^{-3}$ M) and much lower inside neurons ($<10^{-7}$ M). During activity, voltage-dependent Ca^{2+} channels, primarily in nerve terminals and dendrites but also along axons, open and Ca^{2+} enters neurons, thus decreasing the concentration in the extracellular space. With repetitive stimulation, Ca^{2+} may decrease to about 50% (Coles and Orkand, 1985;) or even increase by up to 80% under some conditions (Ziegler and Walz, 1989). In pathological states, such as seizures and ischemia, Ca^{2+} may fall to very low values (Heinemann et al., 1977; for review see Sykova, 1992).

Protons. The extracellular pH of the nervous system undergoes changes as a result of nervous activity, and these have been monitored with pH-sensitive microelectrodes (Chesler, 1990; Chapter 14, this volume). The changes may be monophasic, biphasic, and even triphasic. Recorded swings are about 0.2 pH units during normal activity and up to 0.8 pH units during anoxia or spreading depression (von Hanwehr et al., 1986; Sykova, 1992). Such changes can have measurable effects on the excitability of neurons and the permeability of ion channels (Konnerth et al., 1987), and can modify glutamate receptors (Traynellis and Cull-Candy, 1990; Vyklický Jr. et al., 1990).

A Variety of Substances Are Released from Active Neurons

The composition of the interstitial fluid separating neurons and glia is determined by the secretion of the capillaries that constitute the blood-brain barrier (Cserr, 1988), the secretion of cerebrospinal fluid by the choroid plexus (Neuvelt, 1989), and the metabolic and transport activities of the neurons and glia. Because the fluid is contained in narrow sheetlike spaces, only a few hundred Å wide, it is not easily sampled for analysis. The cerebrospinal fluid is in diffusion equilibrium with the interstitial fluid and

contains a large number of substances which could potentially function as a signal to the glial cells. These include, in addition to the ions discussed above, a wide variety of amino acids, hormones, peptides, catecholamines, organic acids, amines, purines, nucleosides, and metabolites of neurotransmitters and glucose (Siegel et al., 1989). There is some evidence that neurons release amino acids (Weinreich and Hammerschlag, 1975) and even ATP (Lloyd and Stone, 1983) at nonsynaptic regions, and these substances can have effects on glial cells. What is not known is which of these substances are released from active axons and reach the glial cells in sufficient concentration to serve as neuron-glial signals or affect glial activity. The production or release of growth factors responsible for proliferation of oligodendrocyte precursor cells by nerve impulses has been suggested in studies of the developing rat optic nerve (Barres and Raff, 1993). The particular anatomical relations (Figure 31-1) between glial cells and neurons in synaptic regions or at nodes of Ranvier provide loci for substances released from the neurons to react with glial receptors (see Section IV, *Receptors*). Such reactions are very likely to occur. However, in no case can we ascribe a specific physiological function to these receptor-mediated effects. For example, glutamate (100 μM) stimulates intercellular Ca^{2+} waves in cultured astrocytes (see Chapter 17), which propagate through glial gap junctions, and it has been suggested that such waves might originate in the region where astrocyte processes are close to glutamate synapses (Cornell-Bell et al., 1990). Their occurrence under physiological conditions and the functional role of these waves remain to be clarified.

NERVE IMPULSES ARE DETECTED BY GLIAL CELLS

Astrocytes Are Depolarized by Potassium Ion Accumulation Following Nerve Impulses

In the course of a study of electrical properties of spinal cord motor neurons, Coombs et al. (1955) occasionally recorded large resting potentials (−70 mV) from cells that could not be activated either synaptically or by depolarizing currents. They concluded that "the electrode may in these cases have become lodged in nonneuronal cells, possibly large neuroglial cells." Subsequently, it was found that these "idle" cells were depolarized during intense neuronal activity accompanying seizures caused by metrazol (Sugaya et al., 1964). A depolarization of glial cells by massive nerve stimulation in the optic nerve of amphibia (Figure 31-2) was observed by R. Orkand et al. (1966). As the optic nerve of *Necturus* consists only of one kind of ependymoglial cell and small unmyelinated axons, they suggested that the depolarization was recorded from the glial cells and confirmed this hypothesis by injecting a glial cell with aniline blue by electrophoresis through a microelectrode (Kuffler et al., 1966). Moreover, they demonstrated the K^+ electrode-like behavior of the glial membrane, and they further suggested, based on the known release of K^+ during the nerve impulses, that the glial depolarization was the result of K^+ accumulation in the narrow clefts that comprise the extracellular space (R. Orkand et al., 1966). Figure 31-3 illustrates the depolarization of optic nerve glial cells, which follows natural stimulation of the retina with light. The accumulation of K^+ in the extracellular space of the amphibian central nervous system was subsequently measured directly with K^+-selective electrodes (for review see Syková, 1983) and correlated with the glial depolarization (Syková and Orkand, 1980; Chapter 20, this volume). The original observation in 1966, of a depolarization, was extended by Ransom and Goldring (1973), who observed a hyperpolarization of glial cells in the mammalian cerebral cortex following high-frequency direct cortical stimulation. They attributed this effect to a decrease in extracellular K^+, resulting from activity of the neuronal Na^+ pump. This suggestion was supported by measurements with K^+ ion-selective electrodes of a delayed subnormal phase of extracellular K^+ following the initial accumulation (Kriz et al., 1975).

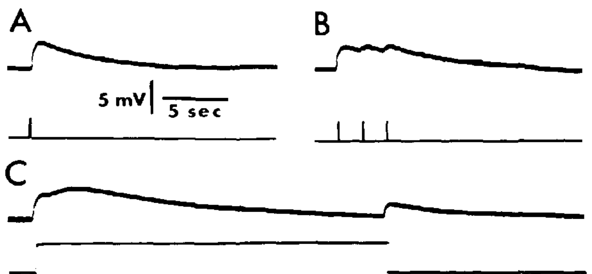

FIG. 31-3. Effect of "natural" nerve activity on the glial membrane potential. Light flashes were shone into the eye while recording from a glial cell in an anesthetized *Necturus*. (A) A single light flash of 100 ms sets up a transient depolarization. (B) The same flash repeated three times. (C) Light stimulus was maintained for 27 seconds. The initial glial depolarization declines due to adaptation of sensory receptors. When the light is turned off, an additional depolarization is produced. [From R. Orkand et al. (1966), with permission.]

Thus, there is good evidence that the membrane potential of glial cells follows changes in extracellular K^+ resulting from neuron activity (R. Orkand et al., 1966; Ransom and Goldring, 1973) or activation of the glial electrogenic Na^+ pump (Tang et

al., 1980). The latter hyperpolarizing effect can be demonstrated by loading the glial cells with Na^+, by bathing the cell in low K^+ solutions for a few minutes, and then increasing the extracellular K^+ to normal levels. On the other hand, there is no evidence that the glial membrane potential in the intact nervous system is affected by the release of other substances from neurons other than in the very special case of the pituitary pars intermedia (Mudrick-Donnon et al., 1993). As a large number of substances have been demonstrated to affect the glial membrane potential (see Chapters 21 to 25), it can be expected that such effects will be demonstrated as adequate techniques become available.

As shown in Figure 31-4, an increase in extracellular K^+ does have an effect on glial metabolism (P. Orkand et al., 1973; Salem et al., 1975) and has been regarded as a signal from active neurons to the surrounding glial cells (Pentreath, 1982; see also Chapter 52, this volume). These records of a decrease in glial cell fluorescence, resulting from a relatively modest increase in extracellular K^+, indicate a transient and reversible oxidation of NADH to NAD. This oxidation could come about as a result the activity of a variety of enzymes involved in brain glycolysis and could play a role in maintaining levels of ATP under anaerobic conditions (Siegel et al., 1989).

Changes in glial membrane potential represent only one indication of possible signal transfer. Neuron-glial signals, however, may result from interactions between substances released from neurons and glial receptors with a subsequent change in the metabolism of the glial cell which is not mediated or accompanied by a change in the ionic permeability pattern of the glial membrane.

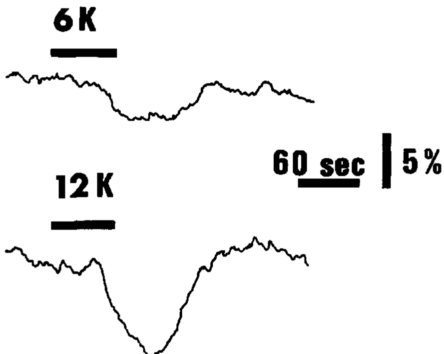

FIG. 31-4. Changes in glial metabolism produced by increased extracellular K^+. Two months after enucleation, the axons in the *Necturus* optic nerve are degenerated and the nerve consists solely of ependymoglia. When such a "glial" nerve was illuminated with light at 355 nM and the emitted light was recorded by a photomultiplier at 480 nM, the amount of light recorded is proportional to the level of reduced pyridine nucleotide (NADH) in the glial cells. Increasing extracellular K^+ from 3 mM to 6 or 12 mM produced a decrease in fluorescence suggesting an oxidation of NADH to NAD. [From P. Orkand et al. (1973), with permission.]

Nerve Impulses Facilitate Glial Voltage-Dependent Channels

In the frog optic nerve, a central nervous system tract that consists of 97% unmyelinated and 3% myelinated axons, oligodendrocytes, and astrocytes, a volley of nerve impulses facilitates voltage-dependent sodium channels in the superficial astrocytes (Marrero et al., 1989). The facilitation results from of a shift of the current voltage curve for activation of the inward sodium current to more hyperpolarizing potentials. Its maximum is about 50 ms after a volley and lasts for about a second. The facilitation is much briefer than the duration of K^+ depolarization, which results from K^+ lost from the axons following passage of the impulse (Figure 31-5). One possible explanation for the facilitation is that the nerve impulses alter the ionic composition of the extracellular fluid, for example, by producing a fall in extracellular calcium, and this ionic change shifts the current voltage curve for activation of the glial channels. This hypothesis suggests that the facilitation should be decreased in high calcium solutions or if calcium entry into the active axons is blocked by addition of calcium channel blockers. Recent studies suggest that, even though the facilitation is blocked by high calcium solutions, a decrease in extracellular calcium produced by axon impulses is not the cause of the facilitation (Marrero and Orkand, 1993). An alternative explanation is that some substance released from the active axons alters the function of the glial channels. Also intriguing, is the possible physiological role of nerve impulses in facilitating glial voltage-dependent channels. First, however, it will be necessary to ascribe some functional significance to the presence of these channels in glial cells (Chapter 12, this volume).

Axon-Schwann Cell Signaling in Squid

An effect of nerve impulses in the squid giant axon on the membrane potential of the surrounding Schwann cells was first reported more than 20 years ago (Villegas, 1972). Hyperpolarization, of the membrane, lasting about 10 minutes, was measured using a technique of repeated transient sampling with microelectrodes penetrating into the tessellated adaxonal Schwann cell, which has an average thickness of less than 2 μm. Using this technique, a complex picture of axon-Schwann cell interactions has evolved (Evans et

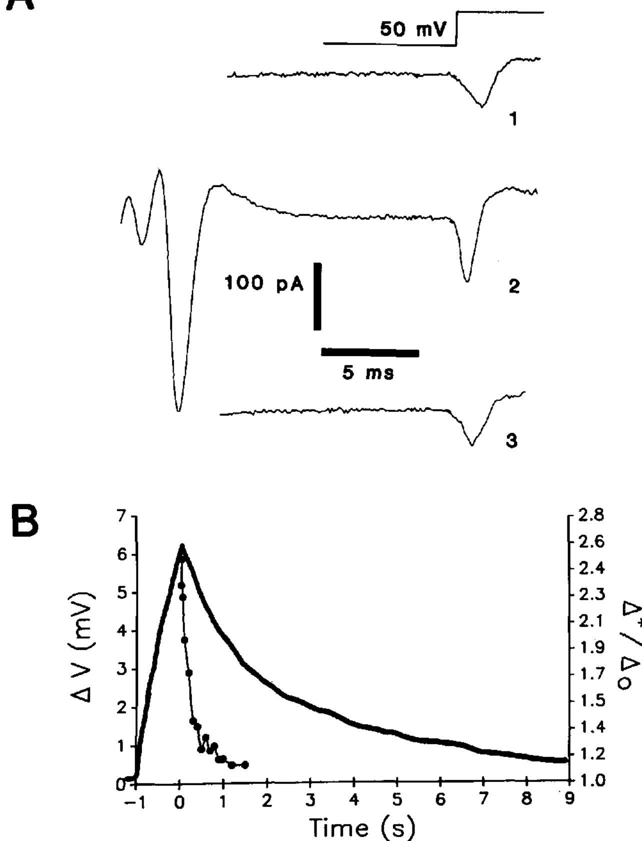

FIG. 31-5. Facilitation of voltage-gated ion channels in frog neuroglia by nerve impulses. (A) *Top*: Voltage-dependent current recorded via a loose-patch clamp electrode in response to a 50 mV depolarizing voltage step applied to the glial surface of a frog optic nerve. *Middle*: The axons of the optic nerve were stimulated 15 ms prior to the same voltage step and the axon action currents are recorded across the resistance of the bathing solution. The voltage-dependent currents from the glial surface are facilitated. *Bottom*: Control current 1 minute after the nerve stimulus was turned off. (B) Time-course of facilitation compared with the depolarization of the glial membrane produced by nerve impulses. The filled circles indicate the facilitation (ratio of facilitated to control response on right-hand scale) following a train of impulses at 10 Hz for 1 second. The heavy line is a record of the glial depolarization (left-hand scale) recorded from a glial cell with an intracellular electrode following an identical train of impulses in a separate experiment. The facilitation of voltage dependent currents recorded from the glial surface decays much more rapidly than the glial depolarization resulting from K^+ accumulation. [From Marrero et al. (1989), with permission.]

al., 1991). According to the proposed scheme, nerve impulses lead to the release of glutamate along the axon, which activates three distinct classes of glutamate receptors on the Schwann cell, that is, rapid and slow *N*-methyl-D-aspartate (NMDA)-type receptors and a quisqualate/kainate (Q/K)-type receptor. Activation of the Q/K receptor leads to corelease of acetylcholine and a vasoactive intestinal polypeptide (VIP)-like peptide from the Schwann cells. The effect of corelease is supposedly subject to modulation by octopamine. The role of NMDA receptor activation is still undefined. The entire scheme encompasses changes in Schwann cell calcium and cyclic AMP, which could alter the cell's behavior. However, the physiological role of this axon-Schwann cell interaction is not apparent. Hyperpolarization of the Schwann cell would favor the uptake of potassium into the cell and thereby aid in the proposed role of these cells to decrease the accumulation of potassium in the intercellular space following axon impulses (Lieberman, 1991). Newer electrophysiological techniques, including the use of patch-clamp electrodes, should provide more precise information concerning the time-course, mechanism, and significance of this axon-Schwann cell interaction.

Axon-Oligodendrocyte Signaling

There is at least one suggestion of a short-lived calcium- and potassium-dependent interaction, detected by voltage-sensitive dyes, between the axon and the oligodendrocyte in the region where the oligodendrocyte fingers are in close apposition to the axon membrane (Lev-Ram and Grinvald, 1986). Electrical stimulation of the axon has been shown to evoke a slow optical signal apparently reflecting a depolarization, lasting about 100 ms, of the oligodendrocytes in the paranodal or internodal region. The depolarization of the oligodendrocyte is suggested to result from a calcium-dependent increase in potassium conductance in the axon and the subsequent accumulation of potassium in a confined extracellular space (Berger et al., 1991).

Butt and Tutton (1992) have found that glutamate and GABA (both at 1 mM) depolarize some oligodendrocytes in the intact mouse optic nerve by a few millivolts. However, their study did not determine if the depolarization resulted from ligand-gated channels or electrogenic uptake of these neurotransmitters (Barbour et al., 1991). Moreover, there is no evidence that these transmitters are released in sufficient quantity and accumulate to a concentration that could depolarize glial cells in the intact nervous system. A possible role of these substances in axon-to-oligodendrocyte signaling at the nodes of Ranvier has to be further explored.

NERVE IMPULSES STIMULATE GLIAL TRANSPORT SYSTEMS

In this section, three established functions of glial cells in the homeostasis of the neuronal microenvi-

ronment are briefly outlined. The glial membrane transport systems involved in this regulation, like most homeostatic processes, are mostly reversible negative feedback systems. An increase above normal of K^+, H^+, or some amino acids leads to glial uptake or buffering, and a decrease below normal leads to glial release (Figure 31-6). It has also been suggested that positive feedback can play a role in a glial cell response to intense nervous activity and extracellular acidification (Chesler, 1990; Ransom, 1992). In effect, the systems are organized so that the substance being controlled represents the appropriate signal. The large glial membrane area forming the boundary of the narrow extracellular clefts provides an anatomical locus for the movement of ions and small molecules across the membrane, which enables glial cells to regulate the composition of the extracellular space. Since the glial cell volume is much greater than that of the extracellular space, movements of substances across the glial membrane may produce large changes in extracellular concentrations with relatively small changes in the glial cytoplasm.

Potassium Homeostasis

The accumulation of K^+ in the extracellular space is a normal consequence of electrical activity in neurons (see Chapters 18 and 47). Changes in extracellular K^+ affect the neuronal membrane potential and a variety of physiological behaviors such as the conduction velocity of the action potential, the amplitude of synaptic potentials and the frequency of rhythmic neuronal discharge. If excessive, the accumulation of K^+ can lead to a complete block of action potentials. The relatively high permeability of glial cells to K^+ and their syncytial nature suggested a role for them in K^+ homeostasis (Kuffler 1967), specifically, that glial cells buffer the rise in extracellular K^+. A rise in extracellular K^+ leads to the following glial response:

1. Glial depolarization and spread of depolarization through the glial syncytium. The resulting current leads to a more uniform distribution of the accumulated potassium (Orkand et al., 1966; Gardner-Medwin, 1983; Reichenbach, 1991). This is the so-called *spatial buffering* of potassium (Coles and Orkand, 1983; Karwoski et al., 1989; Chapter 47, this volume).
2. KCl uptake as a result of the requirements imposed by the Donnan equilibrium [the product of the activities of K^+ and Cl^- outside the cell is equal to their product inside the cell (Hodgkin and Horowicz, 1959; Coles et al., 1989)]. This passive KCl and water influx results in swelling of the glial cells (Wuttke, 1990).
3. K^+ and Cl^- uptake based on the driving force provided by the difference between the membrane potential and the equilibrium potential for the ions (Ritchie, 1991; see Chapter 11, this volume).
4. Additional K^+ uptake resulting from stimulation of the electrogenic Na/K-ATPase by a rise in external K^+ (Tang et al., 1980; Chapter 16, this volume).

FIG. 31-6. Some membrane transport systems in amphibian glial cells involved in the homeostasis of the neuronal microenvironment. Two of the systems, KCl uptake and Na/H exchange, are electroneutral. The remaining three, Na/K exchange pump (i.e., Na/K ATPase), Na-glutamate cotransporter, and Na^+-$2HCO_3^-$ cotransporter are electrogenic because more charge is transported in the direction of net transport (stoichiometries show direction of net charge movement and are imprecise). All the processes are entirely reversible depending on the membrane potential, the concentration gradients for the substances involved and the stoichiometry of the reaction. An increased outward movement of an ion has the same effect as a decreased inward movement of that ion. For example, a fall in extracellular pH will favor either (*1*) movement of HCO_3^- out of the cell or (*2*) less HCO_3^- movement into the cell. In either case, changes in cell pH can be countered by other transport mechanisms such as the Na/H exchanger. [From Orkand (1989), with permission.]

Hydrogen Ion Homeostasis

H^+ buffering minimizes changes in pH and provides for the constancy in the chemical composition of the environment necessary for normal brain function (see Chapter 14, this volume; Thomas et al., 1991). Intracellular pH in glial cells is above 7.0, far more alkaline than would be predicted if H^+ were passively distributed. This indicates that there is active regulation of H^+ across the glial membrane. Recent studies have provided evidence not only for intracellular pH regulation in glial cells but also that glial cells contribute to pH homeostasis in the extracellular fluid. Intracellular pH can be regulated in both neurons and glia by an amiloride-sensitive, bicarbonate-independent, neutral Na^+-H^+ antiport (Deitmer and Schlue, 1987; Astion et al., 1989). In addition, glial pH is regulated by an electrogenic Na^+-$nHCO_3^-$ cotransporter which is partially inhib-

ited by the stilbene derivatives SITS or DIDS (Astion et al., 1991; Deitmer, 1991). The stoichiometry is such that more HCO_3^- is transported than Na^+, so that there is a net transfer of negative charge. The result is that HCO_3^- can readily move in and out of glial cells to buffer changes in extracellular pH. Additional support for the hypothesis that glia play a role in the control of extracellular pH is provided by experiments that demonstrate that the buffering power in the extracellular space is decreased by the addition of the carbonic anhydrase inhibitor acetazolamide (Thomas et al., 1991). These results suggest that bicarbonate-dependent extracellular H^+ buffering is linked to Na^+-HCO_3^- cotransport across glial membranes.

A rise in extracellular H^+ leads to the following glial response:

The uptake of Na^+ and HCO_3^- by the electrogenic cotransporter is reduced or reversed so that HCO_3^- leaves the glial cells to buffer the decrease in extracellular pH (Deitmer, 1991, 1992). In effect, glial cells secrete HCO_3^- in response to an acidification of the extracellular fluid.

The ability of glia to buffer the extracellular pH is therefore a consequence of the basic properties of Na^+-dependent electrogenic transporters (Attwell et al., 1993). The activity, both rate and direction, of these transporters depends not only on the transmembrane gradients of the transported ions but also on the membrane potential. Depending on conditions, the glial cell can either increase or decrease the extracellular concentration of the charged species.

Interactions of Potassium and pH Homeostasis

A relatively large increase in extracellular K^+, such as might occur during intense nervous activity is accompanied by extracellular acidification (Kraig and Nicholson, 1978; Syková, 1992). In contrast, glial depolarization with K^+ (Boyarsky et al., 1988; Deitmer and Szatkowski, 1990) or neuronal activity (Chesler, 1990; Ransom, 1992) leads to a depolarization-induced alkalinization. This is because depolarization decreases the efflux of HCO_3^- from the glial cell via the electrogenic Na^+-HCO_3^- cotransporter (Deitmer and Szatkowski, 1990). Chesler (1990) and subsequently Ransom (1992) discussed the possibility that glial cells contribute to the extracellular acidification in a positive feedback manner to reduce neuronal excitability. On the other hand, in the leech ganglion, Deitmer has demonstrated that extracellular acidification *per se,* in the absence of an increase in K^+, leads to an intracellular acidification due to an increase in HCO_3^- efflux by the glial cells. He further suggests that glial cells tend to "muffle" that is, buffer changes in extracellular pH rather than to reinforce them. Thus, it appears that extracellular acid by itself leads to glial secretion of HCO_3^- to buffer the pH decrease, whereas in the event of intense neural activity leading to K^+ accumulation and acidification, the glial cells tend to contribute to the acidification and decrease neuronal excitability. This ability of glial cells to either buffer or contribute acidity reflects the stoichiometry of the Na^+-$n HCO_3^-$ cotransporter and its voltage dependence (Deitmer and Schlue, 1989; Deitmer and Szatkowski 1990, Astion and Orkand, 1989). This appears to be a special property of glial cells not shared by neurons (Thomas, 1988; Deitmer and Szatkowski, 1989).

The glial role in pH homeostasis appears to be directly related to the properties of the electrogenic Na^+-HCO_3^- cotransporter: (1) A decrease in extracellular pH causes the glial cell to secrete HCO_3^- and restore normal pH; (2) A decrease in extracellular pH accompanied by K^+ accumulation causes the glial cell to secrete acid and decrease neuronal excitability.

Amino Acid Homeostasis

Glial cells take up a variety of amino acids via a Na^+-dependent transport system, and these processes are believed to play a role in inactivation of neurotransmitters like GABA (P. Orkand and Kravitz, 1971) and glutamate (Brew and Attwell, 1987; see Chapters 15, 48, and 54, this volume). The glutamate uptake has been the best studied with electrophysiological techniques (Barbour et al., 1991; Wyllie et al., 1991). A picture is emerging whereby glutamate uptake into cells helps to terminate the action of this neurotransmitter and, in addition, keeps the concentration of glutamate in the extracellular space below neurotoxic levels. When glutamate is applied to glial cells, an inward current can be recorded that is abolished by removal of extracellular Na^+. The current is also evoked by glutamate analogues with the pharmacology of glutamate uptake, and it is presumed that the current reflects electrogenic glutamate uptake by a carrier that cotransports Na^+ into the cell.

A rise in extracellular glutamate or other amino acids leads to the following glial response (depending on the type of glial cell): Stimulation of electrogenic Na^+-dependent amino acid cotransport and or activation of glutamate-gated cationic channels (Wyllie et al., 1991). Figure 31-6 illustrates some glial

cell membrane transport processes which function in extracellular homeostasis.

SUMMARY

Nerve impulse activity has a number of effects on glial membrane transport systems that tend to maintain homeostasis in the neuronal microenvironment. In the case of increases in K^+, H^+, and some amino acids, glial membrane transport systems respond appropriately and reduce the concentration of these substances toward resting levels. These are well-established homeostatic functions of glial cells. In addition, intense neuronal activity leads to K^+ accumulation, and the glial cells contribute to an extracellular acidification that tends to reduce neuronal activity. The alkalinization and acidification of the extracellular fluid are consequences of the reversible voltage-dependent behavior of the glial electrogenic sodium-bicarbonate cotransporter. Voltage-dependent glial currents are facilitated by nerve impulses, but the mediator and significance of this effect are obscure. Furthermore, glial cells are exposed to a number of substances released from active neurons, including a variety of neurotransmitters and metabolites. The role of such substances in neuron-glial signaling and the physiological response of the glial cells to these substances needs clarification.

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VI Molecular and Biochemical Mechanisms

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32 Energy metabolism

BERND HAMPRECHT AND RALF DRINGEN

The indispensable major substrates of energy production in the mammalian brain are glucose and oxygen. Nevertheless, brain perfusion experiments have shown that the brain can do quite well without glucose or any other exogenous substrate for two hours, provided oxygen is offered at a sufficiently high concentration (Geiger, 1958). In this case the brain behaves like the legendary Canadian trapper in his log cabin who runs out of wood while surrounded by deep snow. In order to survive, the trapper burns the furniture in his cabin. Similarly, the brain uses up its stores of glycogen and amino acids and metabolizes protein and membrane lipids to generate energy.

This parable highlights what is meant by energy metabolism in this chapter: metabolic processes necessary for the generation of the energy-rich pyrophosphate bonds in adenosine triphosphate (ATP). Such processes include not only the ATP-synthesizing pathways of substrate chain phosphorylation (glycolysis and Krebs cycle) and oxidative phosphorylation but also the metabolism of the energy store glycogen and the generation, from precursors, of compounds suitable as fuel in the oxidative generation of ATP.

The ultimate fuel in the electron transport or respiratory chain is the most abstracted—in the word's original sense—form of reduction equivalents, the electrons. They end up becoming attached to dioxygen to form O^{2-} ions, which after combination with H^+ ions, are disposed of as water. Before being able to run through the cascade of the electron transport chain from the level of negative redox potential to the most positive one of O_2, the electrons need to be stripped of the atoms that accompany them in the fuel compounds. The sequence of stripping of atoms occurs in a hierarchical fashion. This can best be illustrated by the example of the amino acid alanine oxidatively metabolized as a fuel compound. First, the heteroatom N is eliminated. In this case the elimination is brought about by transamination. In other cases, for example, that of serine, well-known alternative mechanisms are used. At any rate, compounds emerge that consist only of carbon, oxygen, and hydrogen, such as pyruvate in our examples of alanine and serine. The subsequent processes, oxidative decarboxylation and the Krebs cycle, are primarily intramolecular redox reactions aimed at the simultaneous elimination of carbon and oxygen as CO_2. Since molecules such as pyruvate do not contain enough oxygen to allow the production of CO_2 by shear rearrangement, additional oxygen atoms need to be acquired from the solvent water. After the elimination of carbon and oxygen, only hydrogen is left in the form of hydride ions stabilized as NADH or $FADH_2$ for their existence in an aqueous environment. Finally, as the reduction equivalents enter the electron transport chain, the last packaging material, the proton, is stripped from the pair of electrons as an H^- ion.

There is no doubt that these metabolic processes are the same in the nervous system as in the rest of the body. However, in the nervous system it is less certain in which types of cells these processes take place. In fact, they may occur partially in one cell type, for example, the astrocyte, and be continued in another cell type, for example, the neuron or the oligodendrocyte. Such metabolic cooperation between adjacent cells, of course, would require intercellular transfer of metabolites. How little attention is still being paid to this aspect of glial function is exemplified by a recent overview article (Barres, 1991).

Those who are working in the field of neural energy metabolism cannot escape the feeling of just having scratched the surface of the underlying problems. The following questions indicate the state of our ignorance: Do some neurons or certain parts of them really lack hexokinase or cytochrome oxidase, and, if so, what are their physiological specialties that would allow such deficiencies? What is (are) the physiological function(s) of brain glycogen? How is the task to generate energy (and the corresponding metabolic pathways) divided up among the cell types of the central nervous system? What kind of signals switch the energy-generating systems from using glucose to using amino acids or fatty acids? What are the transport systems that allow transcellular transport of nutrients and waste products through the capillary endothelial cells and astrocytes?

Hypotheses about the cellular compartmentation of brain energy metabolism can be derived from the anatomical situation. Strategically speaking, astrocytes are located in a key position between the capillaries on the one side and the neurons and oligodendrocytes on the other side. Thus, all nutrients required for energy production such as glucose, oxygen, and amino acids may have to pass through these cells. In view of this and the presence of glycogen in these cells (Cataldo and Broadwell, 1986a), we consider astrocytes as putative storage and processing plants of energy metabolism.

GLYCOGEN

Glycogen Phosphorylase and Synthase

In brain, glycogen is reported to be found in astrocytes. Minor amounts of glycogen have been detected in neurons, choroid plexus epithelial cells, meningeal cells, capillary endothelial cells, and pericytes (Cataldo and Broadwell, 1986a, 1986b). Glycogen phosphorylase (GP), the activity of which determines the breakdown of glycogen, has been reported to be present in almost all cell types, most likely depending on the technique employed (Friede, 1956, 1959a, 1959b; Shimizu and Okada, 1957; Tewari and Bourne, 1962; Gentschev, 1967; Ohanian, 1972).

For obtaining more reliable results, an immunohistochemical method had to be developed. This required the purification of the brain isozyme of glycogen phosphorylase (Reinhart et al., 1990). Of the three isozymes known, the liver type L, the muscle type M, and the brain type B, the latter was the only one that had not yet been purified to apparent homogeneity. Mouse monoclonal antibodies raised against the B isozyme (Hamprecht et al., 1993) cross-reacted with the L and M isozymes.

The results of indirect immunofluorescence studies on astroglia-rich (Hamprecht and Löffler, 1985) and neuron-rich primary cultures derived from newborn and fetal rat brains, respectively, by using this antibody (Reinhart et al., 1990) are described in Chapter 33, which also discusses the immunocytochemical findings obtained with slices of adult rat brain (Ignacio et al., 1990; Pfeiffer et al., 1990, 1992, 1993). During development, appreciable GP activity appears in rat brain around embryonic day 16 and increases steadily thereafter until adult levels of activity are reached by the end of the third postnatal week (Pfeiffer et al., 1993). In addition to its permanent appearance in astroglial and ependymal cells, GP immunoreactivity is transiently expressed in the pia mater and in choroid plexus cells (Pfeiffer et al., 1993). The absence of GP from neurons of the brain areas mentioned above became the more striking, since the enzyme was strongly expressed in the magnocellular neurons of the mesencephalic trigeminal nucleus (Me5) (Pfeiffer et al., 1993).

Vital staining for GP enzyme activity after electrophoretic separation of brain proteins on "native" gels (Davis et al., 1967) demonstrated the presence of two or three bands, depending on the method and animal species used (Delain et al., 1973; Yonezawa and Hori, 1975; Sato et al., 1976; Richter et al., 1983; David and Crerar, 1986; Mayer and Letsch, 1991; Newgard et al., 1991). These bands are likely to correspond to the brain and muscle isozymes of GP. According to Northern blot analysis, rabbit brain contains predominantly the mRNA of the brain isozyme, transiently expresses the mRNA of the muscle isozyme, and has very little mRNA for the L isozyme (Newgard et al., 1989). The cell-type distribution in brain of these isozymes is unknown, with the exception that astrocytes of cerebral- and cerebellar cortex contain the B isozyme (Ignacio et al., 1990).

There is apparently only one report on the cellular localization in brain of the glycogen-forming enzyme glycogen synthase. According to Inoue et al. (1988) the enzyme appears to be present in all brain cells. This would only be compatible with the notion of cellular restriction of GP if one assumed an alternative pathway of degradation, for example by α-glucosidases (Newgard et al., 1991) in the cells lacking GP, that is, in oligodendrocytes and most neurons.

Glycogen Metabolism in Astroglial Cultures

We know close to nothing about the function of brain glycogen. It is conceivable that the functions of the polysaccharide may not be the same in astrocytes, ependymal cells, and neurons. Two theoretically possible functions are paradigmatically outlined by the situations in liver and skeletal muscle. The main purpose of glycogen storage in liver is to prevent the blood glucose level from falling below a certain threshold concentration. Thus, the glucosyl residues of glycogen are preferentially stored for the benefit of cells of tissues other than liver, and therefore need to be releasable as glucose. In contrast, muscle stores glycogen selfishly. All it may release at situations of high demand for energy is the lactic acid formed by glycolytic breakdown of the mobilized glucosyl residues of glycogen.

The unique capacity of liver to release glucose de-

rived from glycogen is due to the presence of glucose 6-phosphatase. The existence of this enzyme in brain is controversial (Nelson et al., 1985). By histochemical techniques such activity has been detected mainly in neurons (Stephens and Sandborn, 1976; Pertsch et al., 1988). Indeed, the first report on the isolation of the membrane-bound enzyme was using brain as a source rather than liver (Anchors and Karnovsky, 1975). However, it appeared disturbing that the glucose 6-phosphate translocase associated with the liver enzyme could not be found in brain (Fishman and Karnovsky, 1986). More recently, in an immunoblot analysis using antibodies against a subunit of the liver enzyme, immunoreactive material could not be detected in brain (Burchell and Waddell, 1991).

The complexity of brain with the intricately interwoven lamellar and neuritic cellular elements at the submicroscopic level does not allow assessment of the contribution of a certain cell type to a metabolic process by studying brain tissue. Such investigations need to be carried out in culture systems of reduced complexity. Ideally such culture consists of only one cell type. In the case of astroglial cells one may be dealing with astroglial-rich primary cultures derived from the brains of newborn rats or mice (Hamprecht and Löffler, 1985). Besides the numerically dominating astroglial cells they mainly contain oligodendroglial, microglial, and ependymal cells (Raff et al., 1979; Hamprecht, 1986; Reinhart et al., 1990). In the case of mouse primary astroglial cultures these accompanying cell types can be eliminated by culturing in a selective medium (Wiesinger et al., 1991).

The fate of the glycogen glucosyl residues was primarily studied in astroglial-rich cultures from mouse or rat brain. In such cultures the breakdown of glycogen can be induced by several hormones (Figure 32-1), among them also some that elevate the level of cyclic adenosine monophosphate (cyclic AMP) (Hamprecht, 1986). Such hormones are adenosine (Magistretti et al., 1986), norepinephrine (Quach et al., 1978; Rosenberg and Dichter, 1987; Cambray-Deakin et al., 1988; Subbarao and Hertz, 1990; Hamprecht et al., 1993), histamine (Quach et al., 1980; Arbones et al., 1990), serotonin (Quach et al., 1982), and vasoactive intestinal polypeptide (Magistretti et al., 1981). Glycogen degradation is also triggered by raising the concentration of Ca^{2+} in the cytosol (Hamprecht et al., 1993). The fact that elevation of the cytosolic levels of either cyclic AMP or Ca^{2+} triggers the catabolism of glycogen (Figure 32-1) points to an involvement of phosphorylase kinase. In addition, withdrawal of glucose (Dringen and Hamprecht, 1992a, 1993a) leads to loss of glycogen: On deprivation of glucose the glycogen content drops in a process exhibiting first-order kinetics with a half-life of 7 minutes (rat) or 15 minutes (mouse).

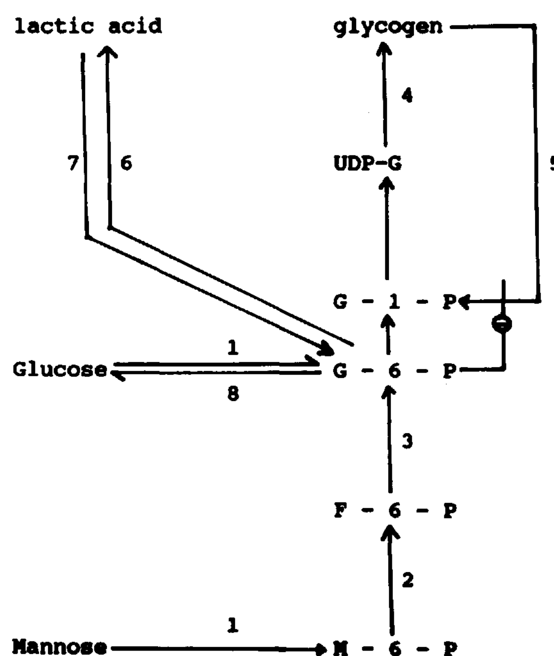

FIG. 32-1. Pathways of synthesis and degradation of glycogen in astroglial cells. *M-6-P*, mannose 6-phosphate; *F-6-P*, fructose 6-phosphate; *G-6-P*, glucose 6-phosphate. The numbers represent enzymes or pathways: *1*, hexokinase (EC 2.7.1.1); *2*, mannose-phosphate isomerase (EC 5.3.1.8); *3*, glucose phosphate isomerase (EC 5.3.1.9); *4*, glycogen synthase (EC 2.4.1.11); *5*, glycogen phosphorylase a (EC 2.4.1.1); *6*, glycolysis; *7*, gluconeogenesis; *8*, glucose 6-phosphatase (EC 3.1.3.9).

The degradation following deprivation of glucose is most likely a consequence of the loss of glucose 6-phosphate. This compound (Mayer and Letsch, 1989) and 2-deoxyglucose 6-phosphate (Dringen and Hamprecht, 1993b) block phosphorylase (Figure 32-1), whereas the unphosphorylated sugars do not. Feeding of 2-deoxyglucose to astroglial cells gives rise to the corresponding 6-phosphate, which is practically not metabolized and thus affords a permanent block of glycogenolysis (Dringen and Hamprecht, 1993b). The inhibitory action of deoxyglucose 6-phosphate on glycogen phosphorylase may also explain that deoxyglucose in the culture medium of astroglial cells elevates the cellular content of glycogen (Swanson et al., 1989). On the other hand, a similar effect of methionine sulfoximine on astroglial cells in brain (Folbergrová, 1973) and in culture (Swanson et al., 1989) remains unexplained, although an action on the level of glycogen phosphorylase has been suggested (Swanson et al., 1989). Also the molecular mechanism underlying similar effects of aspartate and glutamate (Swanson et al., 1990) are unknown. 1,5-Gluconolactone is another compound that can block glycogen phosphorylase

(Gold et al., 1971; Tu et al., 1971), and also blocks glycogen mobilization in astroglial cells (Dringen and Hamprecht, 1993b). Due to the spontaneous hydrolysis of the lactone and the lack of inhibitory capacity of the ensuing gluconate, the block exerted by the lactone is only transient. Both 2-deoxyglucose, as a precursor of deoxyglucose 6-phosphate, and gluconolactone may turn out as useful tools in the analysis of the glycogen metabolism in brain. Indeed, if glycogen degradation is blocked by 2-deoxyglucose or gluconolactone, the amount of lactate released from astroglial cells into the culture medium decreases by the amount expected to be generated from the glycogen present (Dringen and Hamprecht, 1992b).

After depletion by withdrawal of glucose, glycogen is restored in the astroglial cultures within 2 hours of repletion of glucose (Dringen and Hamprecht, 1992a). The maximal level of glycogen is twice as high if also serum, insulin or insulinlike growth factor I (IGF-I) is present. The latter is at least one order of magnitude more potent than insulin (Dringen and Hamprecht, 1992a). Therefore, and in view of the fact that brain contains a truncated variant of IGF-I (Carlsson-Skwirut et al., 1986), which binds more strongly to IGI-I receptors of brain membranes than IGF-I itself (Carlsson-Skwirut et al., 1989), it is safe to state that it is likely an IGF-I that would exert an elevation of glycogen levels in brain cells. This is especially interesting, since astroglial cells in culture produce IGF-I and express receptors for it (Ballotti et al., 1987), which would allow an autocrine effect. A report that insulin (1 μM) increases the incorporation of [^{14}C] glucose into cultured astroglial cells by 60% (Kum et al., 1992) is in accord with the results mentioned above (Dringen and Hamprecht, 1992a).

The mechanism by which insulinoids bring about an increased level of glycogen has been elucidated. The receptor tyrosine kinase phosphorylates the regulatory subunit of type-1 protein phosphatase, which enzyme, in turn, activates glycogen synthase by dephosphorylation (Dent et al., 1990).

Astroglial glycogen can not only be synthesized from glucose (Figure 32-1). Refeeding depleted cells with mannose instead of glucose leads to the same cellular concentration of glycogen as in glucose-fed cultures (Dringen et al., 1994a). Mannose is transported into the cells by the cytochalasin B-sensitive glucose transporter and is phosphorylated by hexokinase (Bergbauer et al., 1993). The ensuing mannose 6-phosphate is isomerized to fructose 6-phosphate by mannose-6-phosphate isomerase. With the further enzymatic isomerization to glucose 6-phosphate the carbohydrate moiety has entered the glycogenic pathway of glucose. Compatible with this notion is that the astroglia-rich cultures contain mannose 6-phosphate isomerase and can convert mannose to lactate (Bergbauer et al., 1993; Dringen et al., 1994a) (Figure 32-1).

Mannose is not normally a constituent of blood (Hawkins and Mans, 1983; Akazawa et al., 1986). Why then should astroglial cells contain mannose 6-phosphate isomerase? Possible they need the enzyme in the processes of synthesis and breakdown of glycoproteins. In this case it is expected, but not known, to be present in all cell types of the brain. On the other hand, the high specific activity of the enzyme in astroglia-rich cultures, higher than that of hexokinase (Dringen et al., 1993c), and the appreciable capacity of brain to metabolize mannose to lactate and CO_2 (Chain et al., 1969; Sloviter and Kamimoto, 1970) point to a metabolically more demanding function than would be required in the turnover of glycoproteins. In contrast to glucose or mannose, astroglial cells do not at all, or only slightly, accumulate glycogen if they are grown in the presence of galactose and fructose, respectively, instead of glucose (Dringen and Hamprecht, 1993a).

Astroglial Cells as a Lactic Acid Store

The most important aspect of glycogen degradation is the fate of the glucosyl residues. Surprisingly, the glucosyl residues do not appear as free glucose released into the surrounding incubation medium. Rather, the cells release lactic acid (Dringen and Hamprecht, 1992b; Dringen et al., 1993a) (Figure 32-1). If glucose had been excreted it would have been detected, as was the case with cultured hepatocytes.

The rapid degradation and repletion of glycogen, that is, the high turnover of the storage polysaccharide, can be analyzed by enzymatic determination of glycogen and can also be demonstrated by the use of radioactively labeled glucose (Hamprecht et al., 1993). As a consequence of a drop in the concentration of extracellular glucose or of an adequate hormonal stimulus, glycogen may be broken down locally. The glycolytic processing of the glucosyl residues benefits the astrocyte only to a small extent. The bulk of the free energy of combustion, however, is likely to be passed on to the neighboring neurons and oligodendrocytes within the lactic acid generated (Figure 32-2).

The appearance of lactate rather than glucose as a consequence of glycogen breakdown allows us to consider astroglial glycogen as a store of lactic acid rather than of glucose. The fact that the two lactic

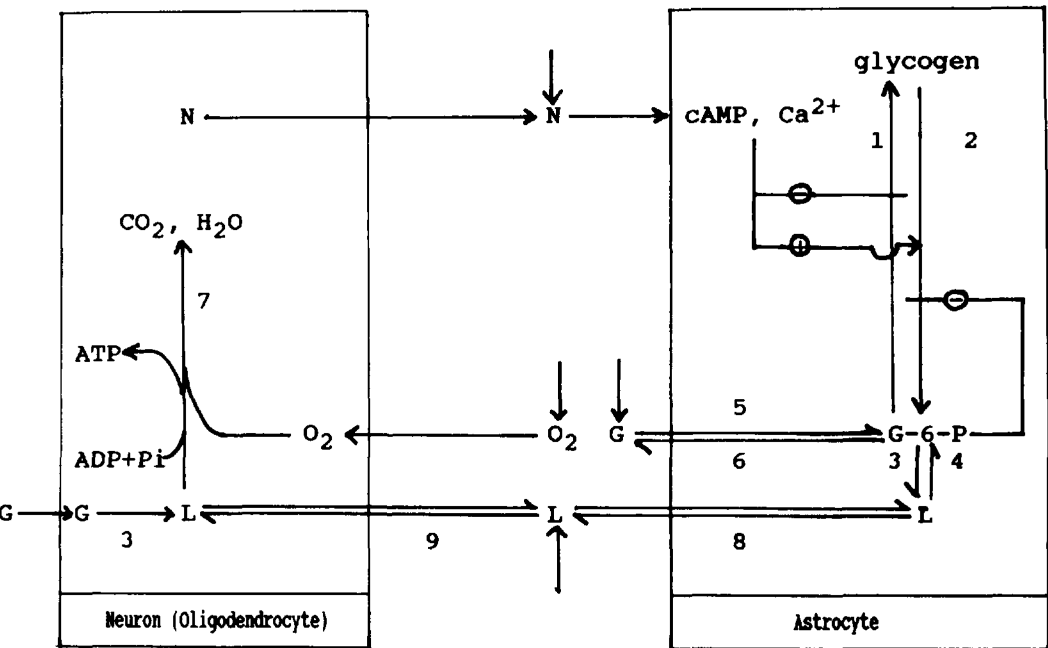

FIG. 32-2. Hypothetic scheme of metabolic interactions between brain cells due to dynamics of astroglial glycogen and lactate. *L*, lactic acid or lactate; *G*, glucose; *G-6-P*, glucose 6-phosphate; *N* neurohormone, neurotransmitter. The numbers represent pathways or enzymes: *1*, glycogenesis; *2*, glycogenolysis; *3*, glycolysis; *4*, gluconeogenesis; *5*, hexokinase; *6*, glucose 6-phosphatase; *7*, oxidative metabolism of lactate, including the tricarboxylic acid cycle and oxidative phosphorylation; *8* and *9*, astroglial and neuronal lactate transport, respectively. The *arrows* outside the cells indicate exogenous supply of a substance, either from outside the brain (or in the case of *N*, from another neighboring neuron). The production of ATP during glycolysis has been omitted for the sake of clarity. The interaction shown is between an astrocyte on the one side and a neuron or oligodendrocyte on the other side. In the case of an oligodendrocyte being the partner, it is not known whether it can release a neurohormone that could induce (second messenger-mediated) glycogenolysis in the astrocyte. If the concentration of extracellular glucose drops or if neurohormones emitted by neighboring neurons elevate the level of second messengers in the cytosol of the astrocyte, glycogen breakdown is initiated and glycogen synthesis is stopped. The *G-6-P* generated is used for glycolytic energy production. The lactate produced is released from the astrocyte and taken up into a neighboring neuron or oligodendrocyte (or even microglial cell?) to be used for energy production by oxidative metabolism. If the cell or part of it can also perform glycolysis, ATP can also be generated by using this pathway. Thus, if this neuron (or other neurons in the vicinity) is excited, the neurohormone released may be "understood" by the astrocyte as a "request" for delivering an energy substrate. Instead of lactate, other energy substrates such as pyruvate or ketone bodies might be generated and forwarded as well. It is conceivable that momentary local conditions can make lactic acid available to astrocytes from neighboring cells or their processes. The astrocytes might then dispose of the lactic acid by gluconeogenesis.

acid molecules are almost as valuable a fuel material in the oxidative pathways of generation of energy as the glucose they are derived from, is illustrated by the capacity of isolated rat brain cells (Vicario et al., 1991) or brain slices (Schurr et al., 1988) to use lactate as a source of energy. The observation that fetal sheep brain does not take up exogenously offered lactate (Harding and Charlton, 1990) may not be a contradiction, since some brain cells (neurons, oligodendrocytes) may well be using endogenous lactate produced by and released from their neighbors, for example, astrocytes. That such local production of lactate is a physiologically important phenomenon in the central nervous system has been clearly demonstrated by *in situ* microdialysis studies of the brain (Kuhr and Korf, 1988; Korf, 1989; De Bruin et al., 1990) and by work carried out on isolated retina (Ames et al., 1992). In culture the astroglial cells are taken out of the context of their physiological neighbors (neurons, oligodendrocytes, microglia). Thus, the lack of extracellular appearance of glucose on degradation of glycogen in cultured astroglial cells does not necessarily mean that this should also be the case in brain. *In situ* microdialysis studies are required to clarify this point.

Thus far, the most clear-cut example of metabolic cooperation between neural cells has been the interaction between glial and photoreceptor cells in the retina of the honeybee drone (Tsacopoulos et al., 1987, 1988). The glial cells store glycogen but lack mitochondria, whereas it is the other way around for the photoreceptor cells. On illumination, the glial cells receive from the photoreceptor cells a signal of hitherto unknown nature, upon which they degrade

their glycogen and provide the photoreceptor cells with a metabolic substrate that is neither glucose nor lactate or pyruvate. The photoreceptor cells use this compound and O_2 for the generation of energy. Such metabolic coupling is likely to exist also in the mammalian central nervous system. After all, oligodendrocytes (Kao-Jen and Wilson, 1980; Snyder and Wilson, 1983) and some neurons (Kao-Jen and Wilson, 1980; Simurda and Wilson, 1980; Katoh-Semba et al., 1988) contain hexokinase only at low activity and thus must widely rely on oxidative generation of energy. Important in this context is the protection granted by astroglial cells to neurons in culture during anoxia (Vibulsreth et al., 1987). With their stores of glycogen, astroglial cells in culture also enable neighboring neurons to survive a deprivation of glucose (Swanson and Choi, 1993).

Neural Transport and Utilization of Lactate

The concept that lactate released from astrocytes may be utilized by neurons implies the transport of lactate across the plasma membranes of these two cell types (Figure 32-2). It has been shown that the lactate produced by astroglial cells can not only be derived from glycogen but also from glucose (Pauwels et al., 1985; Walz and Mukerji, 1988a, 1988b). From studies of cellular release of lactate no data can be obtained that would allow conclusions as to the mechanism involved. Therefore, inward transport has to be investigated. According to the principle of the microscopic reversibility of chemical reactions, the data obtained should allow the construction of a mechanism also for the outward transport. The transport of lactate into cultured astroglial cells is quite rapid, even more so if the cells already contain lactate at the onset of the transport experiment and if the pH of the incubation medium is low (Dringen et al., 1994b; Tildon et al., 1993). Due to the rapidity of lactate uptake, using cells attached to culture dishes does not allow a reliable time resolution of the uptake process in the range below half a minute. This situation bars the determination of meaningful kinetic constants for the uptake process (Dringen et al., 1994b). Nevertheless, such constants have been published and the existence of a high-affinity/low-capacity and a low-affinity/high-capacity transport system has been postulated (Tildon et al., 1993). No such suitable carrier systems could be detected in another study, the data of which led to the conclusion that the rate of lactate uptake into astrolia-rich primary cultures derived from rat brain is proportional to the lactate concentration in the incubation medium (Dringen et al., 1994b). In other words, the transit of lactate, possibly as lactic acid, through the plasma membrane is facilitated by a nonsaturable pore or channel, analogous to that allowing the permeation of sorbitol (Stahl et al., 1989). Encouragement to accept this conclusion is lent by the notion that a widely employed inhibitor of lactate and pyruvate transport, α-cyano-4-hydroxycinnamate (Halestrap and Denton, 1974), fails to inhibit lactate uptake into astroglial cells (Dringen et al., 1994b).

If the lactate released from astrocytes is to be used as fuel material in neurons and oligodendrocytes (Figure 32-2), or in some regions of these cells, it must be taken up into them. Indeed, they can utilize lactate as energy source, at least in culture (Edmond et al., 1987). The uptake of lactate into neuron-rich cultures derived from fetal rat brains consists of two components, a saturable component and a nonsaturable component, of which only the former can be completely blocked by α-cyano-4-hydroxycinnamate (Dringen et al., 1993c). This contrasts with the lack of a saturable and inhibitable transport system in astroglial cells (see above; Dringen et al., 1994b) and resembles the situation of glucose transport in astroglial cells, in which only the saturable component would be inhibited by cytochalasin B (Stahl et al., 1989).

All cells that contain hexokinase should be able to generate ATP glycolytically, and all cells expressing cytochrome oxidase can be assumed to have the capacity of producing ATP by oxidative phosphorylation. Histochemical and activity studies of the distribution of these enzymes (Wilkin and Wilson, 1977; Kao-Jen and Wilson, 1980; Simurda and Wilson, 1980; Snyder and Wilson, 1980; Sandell, 1984; Aoki et al., 1987; Katoh-Semba et al., 1988; Borowsky and Collins, 1989; Hevner and Wong-Riley, 1989; Bilger and Nehlig, 1991; Burgess and Wilson, 1991; Gonzales-Lima and Garrosa, 1991; Griffin et al., 1992; Karmy et al., 1991) in the central nervous system revealed that their "territories" overlap only partially. This means that some cells rely on either the one or the other pathway of production of energy, whereas others have an alternative and, therefore, can more flexibly respond to a changing demand of energy. In the case of expression of both pathways in a cell type (e.g., astrocyte), the ratio of their maximal activities may vary with the stage of development and the anatomical location. Due to their juxtaposition to the capillaries, the astrocytes are closer to the source of glucose. In view of the normal abundance of glucose it would make sense (1) that astrocytes relied more strongly on glycolysis than on oxidative phosphorylation, and (2) that neurons and oligodendrocytes would preferentially pro-

duce their energy by oxidative phosphorylation. Their remote location in respect to the blood as a source of substrates for the production of energy requires a more efficient utilization of the energy substrate. This strategy is facilitated by the rapidity with which the very low molecular weight hydrophobic compound O_2 can diffuse transcellularly. The observation that astroglial cells are more dependent on glucose than neurons (Kauppinen et al., 1988) is compatible with this consideration. Those neurons that contain hexokinase can more rapidly generate ATP than would be possible by oxidative phosphorylation, and thus they would be able to respond more instantaneously to sudden demands for energy.

GLUCONEOGENESIS

Classical biochemistry has shown that in mammals gluconeogenesis is a privilege of liver and kidney. The term means generation of glucose from gluconeogenic substrates such as lactate, pyruvate, and a wide variety of amino acids including alanine, serine, threonine, aspartate, and glutamate. In the physiological practice of a starving animal the liver extracts such a substrate from the blood and, at the expenditure of 6 ATP (in the cases of alanine and lactate), synthesizes glucose from it, which is released into the bloodstream. For the reversal of lactic acid fermentation seven enzymes of glycolysis are used plus the four key enzymes of gluconeogenesis: pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase (FBPase) and glucose 6-phosphatase (G6Pase). However, it should be stressed that, probably with the exception of FBPase, these enzymes can be involved in other pathways as well. Thus, PC also acts as an anaplerotic enzyme, which—by catalizing the formation of oxaloacetate from pyruvate—replenishes the pool of Krebs cycle intermediates. By catalyzing the reverse reaction to its gluconeogenic activity, PEPCK may serve the same purpose as PC, that is, the formation of oxaloacetate from phosphoenolpyruvate and CO_2. In fact, PEPCK occurs in nongluconeogenic tissues, such as muscle, and may function anaplerotically (Schöttler and Wienhausen, 1981). G6Pase can play a role also in the last step of glycogenolysis. To our knowledge, only for FBPase has an alternative function thus far not been suggested.

The prerequisite for gluconeogenesis from lactate is the presence of all four key gluconeogenesis enzymes (Figure 32-3). Gluconeogenesis from amino acids that are degraded to Krebs cycle intermediates

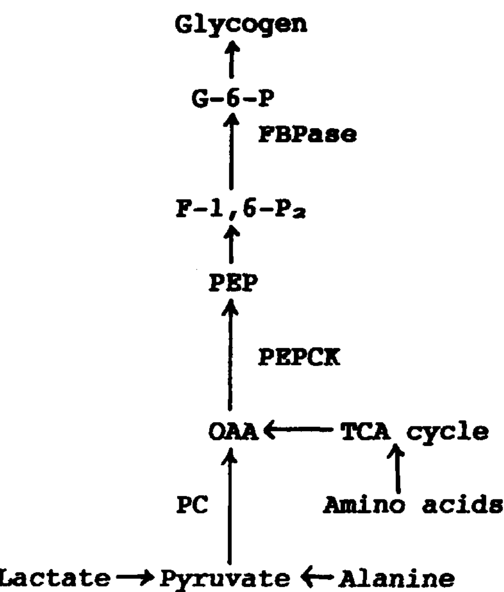

FIG. 32-3. Scheme of gluconeogenesis in astroglial cells. Numerous intermediate enzymatic steps have been omitted for the sake of clarity. *PC*, pyruvate carboxylase (EC 6.4.1.1); *TCA*, tricarboxylic acid; *OAA*, oxaloacetate; *PEP*, phosphoenolpyruvate; *PEPCK*, phosphoenolpyruvate carboxykinase (EC 4.1.1.32); *F-1,6-P$_2$*, fructose 1,6-bisphosphate; *G-6-P*, glucose 6-phosphate; *FBPase*, fructose 1,6-bisphosphatase.

does not require PC any more. Nevertheless, the occurrence of the four key gluconeogenic enzymes in an organism does not guarantee the occurrence of gluconeogenesis. It is a paradoxical situation that a blood-dwelling, parasitic helminth contains these enzymes, although it is living in the presence of an abundance of glucose in blood. Since the animal is unable to carry out gluconeogenesis (Tielens et al., 1991), this set of enzymes is in search of a function.

Two lines of evidence made it likely that gluconeogenesis could also occur in brain. One of these is the presence in brain of the gluconeogenic enzymes PC (Yu et al., 1983; Shank et al., 1985; Faff-Michalak and Albrecht, 1991), PEPCK (Zimmer and Magnuson, 1990), FBPase (Phillips and Coxon, 1975; Majumder and Eisenberg, 1977; Hevor and Gayet, 1978, 1981; Carter, 1983; Hevor et al., 1985; Chattoraj and Majumder, 1986; Liu and Fromm, 1988), and G6Pase (Anchors and Karnovsky, 1975). Also the fixation by astroglial cells of $^{14}CO_2$ in acid-stable products can be taken as support of the existence of PC in brain cells (Kaufman and Driscoll, 1992). In the case of PEPCK other authors (Wiese et al., 1991) have been unable to detect the enzyme in brain. The second line of evidence is dealing with the synthesis in tissue of the central nervous system of glycogen glucosyl residues from gluconeogenic precursors (Ide et al., 1969; Phelps, 1975; Phillips and Coxon, 1975; Goldman and Witkovsky, 1987; Goldman, 1988).

This creates a semantic problem with the definition of gluconeogenesis given above. We would have to redefine this term by specifying that we understand by gluconeogenesis the synthesis from gluconeogenic precursors (classically lactate) of glucosyl residues (rather than free glucose). The biochemical consequence of this change in semantics would be that G6Pase is not necessarily required any more for gluconeogenesis (Figure 32-3). Consequently, in this context we would no longer have to worry about the above-mentioned weak evidence for the existence of the enzyme in brain.

The occurrence of the key gluconeogenic enzymes in brain would not be sufficient to ensure gluconeogenesis. They would have to coexist in the same cell. This must be the case, since nervous tissue is apparently capable of incorporating label from radioactive precursor molecules into glycogen. Of the key enzymes only PC has been allocated with some certainty exclusively to one cell type, the astroglial cells (Shank et al., 1985; Yu et al., 1983; Cesar and Hamprecht, 1993a, 1993b). Therefore, we hypothesize that astroglial cells are the gluconeogenic cell type of the central nervous system. Indeed, sorbitol-selected (Wiesinger et al., 1991) pure astroglial cultures from mouse brain are capable of incorporating label from [^{14}C]lactate (Dringen et al., 1993b) or labeled alanine aspartate or glutamate (Schmoll and Hamprecht, 1993; Schmoll et al., 1993b) into glycogen. By taking advantage of the specificity of the three enzymes amyloglucosidase, hexokinase, and glucose 6-phosphate dehydrogenase it was proven that the label of lactate had indeed entered the glucosyl residues of astroglial glycogen rather than exclusively the protein accompanying the glycogen particles (Dringen et al., 1993b). It is important to point out that a demonstration of the incorporation of label from [^{14}C]lactate into glycogen was only possible in the presence of glucose. The depletion of glycogen in cells kept without glucose has already been described above. Under physiological conditions the concentration of extracellular glucose will always be well above zero, and gluconeogenesis will be taking place in the presence of glucose (Newgard et al., 1983, and literature cited therein).

With the exception of PC the activity of which is depressed by serum in the culture medium of glial cells (Lopes-Cardozo et al., 1989), the literature provides no insight into the regulation of the gluconeogenic enzymes in brain, not even for PEPCK, the hormonal induction of which in liver cells is quite well understood at the genome level (Granner et al., 1991). Recently it has been reported that PEPCK and FBPase activity in astroglial-rich primary cultures, but not in hepatocyte cultures, are inversely regulated by long-term withdrawal of glucose. In contrast to the strong rise of PEPCK activity elicited in hepatocytes by a cyclic AMP analogue and a glucocorticoid, no such response was encountered in astroglial cultures (Schmoll et al., 1993a). Apparently, gluconeogenesis is differently regulated in hepatocytes and astroglial cells.

The function of gluconeogenesis in the nervous system is enigmatic. The situation has to be explained that the same cells can generate lactic acid and use it for the synthesis of glycogen (Figure 32-1). It may well be that a given astrocyte in brain would rapidly switch between production and consumption of lactate, depending on the activities and needs of its neighboring neurons and the probably concomitant requirements of the adjacent oligodendrocytes. In situations of high rates of generation and release of lactic acid in their proximity, astrocytes could dispose of the deleterious acid by using it up in the gluconeogenic process. This would mean simultaneously serving three purposes, buffering and control of osmolarity of the extracellular fluid and recycling of the "waste product" generated by a neighboring cell during its (electrical) activity.

FATTY ACIDS AND KETONE BODIES

The facts that eicosanoids are also produced in brain and that they are derived from essential fatty acids that have to be supplied to the animal with the food already make clear that the brain must be able to take up such fatty acids (see Chapter 25, this volume). This is indeed the case (Dhopeshwarkar and Mead, 1969; Oldendorf, 1973; Kimes et al., 1983; Pardridge and Mietus, 1980; Rowley and Collins, 1985). Uptake is a prerequisite but not a sufficient criterion for the utilization of fatty acids of various sizes for the generation of energy. There is ample evidence that an exogenously applied radioactively labeled medium-chain fatty acid such as octanoic acid is rapidly metabolized in brain (Rowley and Collins, 1985). There, its carbon atoms quickly label the glutamate-glutamine pool, thus proving that oxidative metabolism has taken place (Cremer et al., 1977).

These results also demonstrate the participation of astrocytes in these events, since glutamine synthetase is predominantly expressed in astrocytes (see Chapter 33, this volume). In fact, cultured astroglial cells were shown to metabolize fatty acids (Bourre et al., 1983; Robert et al., 1983; Edmond et al., 1987). Auestad et al. (1991) have subsequently demonstrated that solely this cell type is capable of metabolizing octanoate and of generating ketone bodies, preferentially 3-hydroxybutyrate, from it. This result

again emphasizes the pivotal role of astrocytes as plants for the production of fuel molecules from exogenous raw material.

Which are the cells that can benefit from these astroglial activities and use the ketone bodies as fuel? Studies on the adult brain led to the conclusion that quantitatively seen, fatty acids are of little importance for energy metabolism of the adult mammalian brain (Carey, 1975; Miller et al., 1987). In contrast, the ketone body 3-hydroxybutyrate is rapidly utilized in the brain, although there exist enormous regional differences (Hawkins and Biebuyck, 1979). These authors consider ketone bodies as fuel supplement to glucose in the normally alimented animal. Under starvation, ketone bodies can become the dominant fuel of the brain (Owen et al., 1967). In cultured oligodendroglial cells, the ketone bodies acetoacetate and 3-hydroxybutyrate can be used as a fuel material for oxidative generation of energy (Sykes et al., 1986; Edmond et al., 1987). Cultured oligodendroglial cells strongly prefer ketone bodies to lactate as substrates for energy production (Sykes et al., 1986). If this held also for the adult brain, it would mean that lactate released from astrocytes would preferentially benefit neurons. Candidate users of lactate could also be microglial cells, the metabolic preferences of which are unknown. The notion that in most classes of brain cells energy substrates alternative to glucose can be utilized is supported by the fact that withdrawal of glucose does not affect the ATP level in cultured neurons and astroglial cells (Pauwels et al., 1985).

AMINO ACIDS

The experiment by Geiger (1958) mentioned earlier indicated that amino acids in free form and in proteins must also be considered fuel material for the brain. For example, leucine is taken up and metabolized by the brain (Cremer et al., 1977). It may well be used for the synthesis of ketone bodies.

Astroglial cells oxidize glutamate to CO_2 (Yu et al., 1982). The pathway is not via transamination to 2-ketoglutarate, since a transaminase inhibitor is without effect. This leaves as a possibility only usage of the alternative pathway via glutamate dehydrogenase (Yu et al., 1982). Since in brain this enzyme is localized in astrocytes, such a pathway of glutamate metabolism would have to be confined to them (Kaneko et al., 1987; Hussain et al., 1989; Würdig and Kugler, 1991). A rapid depletion from culture medium, even in the presence of glucose, of the branched-chain amino acids valine, leucine, and isoleucine has been observed in astroglia-rich cultures (Bixel et al., 1993).

The degradation in brain of amino acids (e.g., glutamate) to a member of the Krebs cycle would require also the activity of an enzyme that can permanently withdraw another member of the Krebs cycle. The most likely candidate for such an enzyme would be malic enzyme (Figure 32-4). Purification of the cytosolic isoform of the enzyme allowed the generation of monoclonal antibodies. In astroglia-rich cultures these antibodies stained GFAP-positive astroglial cells and myelin basic protein-positive oligodendroglial cells. The latter were the most intensively stained cells. Neurons in neuron-rich cultures were not stained (Kurz et al., 1993). This result is backed up by results from studies of enzyme activity in cell homogenates and by Western blot analysis of cell homogenates. The mitochondrial isoenzyme appears to be almost absent from the astroglia-rich cultures. In a previous immunocytochemical study of the cerebellum using antibodies of unknown specificity, the cytosolic malic enzyme was found associated with Bergmann glial cells (Martinez-Rodriguez et al., 1989).

These results are certainly compatible with the idea of astroglial cells as processing plants for the generation of fuel molecules. In the case of malic enzyme action the Krebs cycle intermediate malate would be transformed into pyruvate and CO_2, while NADP would be reduced. The pyruvate could be used locally in oxidative generation of ATP. Alternatively, it could be exported, either unchanged or reduced to lactate or alanine. It is known that alanine can be generated in astroglia-rich cultures from lactate (Dringen and Hamprecht, unpublished results) or pyruvate (Hamprecht and Dringen, 1994). The function of malic enzyme in oligodendrocytes would primarily have to be sought in the generation of NADPH that is required for the synthesis of myelin lipids.

Besides the C-3 compounds produced from amino acid-derived Krebs cycle intermediates with the aid of malic enzyme, these intermediates themselves could also be released by the astroglial cells (Sonnewald et al., 1991) for the benefit of neighboring cells, for example, neurons. The report that synaptosomes have a transport system for 2-ketoglutarate supports this view (Shank and Campbell, 1982).

CONCLUSIONS

The analysis of the widespread involvement of glial cells in the energy metabolism of the brain has generated new working hypotheses that should lead to

more detailed experimental approaches than were possible thus far. The concept emerged that astroglial cells are processing plants for the production of fuel material from fatty and amino acids that can be used by their neighboring cells. They may also remove "waste products," such as lactic acid, released from these neighbors during metabolic activity accompanying neuronal activity. In this context it appears most important that the enzymes characteristic of the various metabolic pathways be immunocytochemically localized in all areas of the central nervous system. This would allow the assignment of metabolic tasks with cell types and the examination of the general validity of such a separation of metabolic tasks among cell types, as far as development and regions of the central nervous system are concerned.

In a next step the *in situ* functioning of such pathways during physiological tasks will have to be analyzed by a combination of physiological, biochemical, and pharmacological investigations as they have been applied in a paradigmatic way by the groups of Tsacopoulos (1988) and Korf (1991). The *in vitro* studies allow us to find out the likely pathways used by certain cell types and thus enable us to ask more precise experimental questions to the brain itself than would be possible without the studies of cultured cells.

The plasticity of the metabolic facilities of the brain cells in response to developmental and acute changes of physiological situations needs to be found out. It is important to obtain information on the susceptibility of the alternative pathways for the generation of fuel molecules to the plethora of neurohormones (growth factors, neurotransmitters).

Much is also to be learned from inborn errors of metabolism and, thus, from collaboration with clinicians. We are presently experiencing a renaissance of interest in brain energy metabolism. This may be due to the fact that the dogmas that have been followed (such as glucose being the only fuel material of the brain) have not withstood the accumulating experimental results. This renaissance is certainly also elicited by the molecular information on the proteins involved that has become available through the techniques of modern molecular biology. It will certainly profit also from the enormous spatial resolution that is being brought about by confocal laser scanning microscopy. No single laboratory will have available all the equipment, expertise, and funds required for the multidisciplinary approach to understanding the functioning of the nervous system. Therefore collaboration will more than ever be the slogan of the future.

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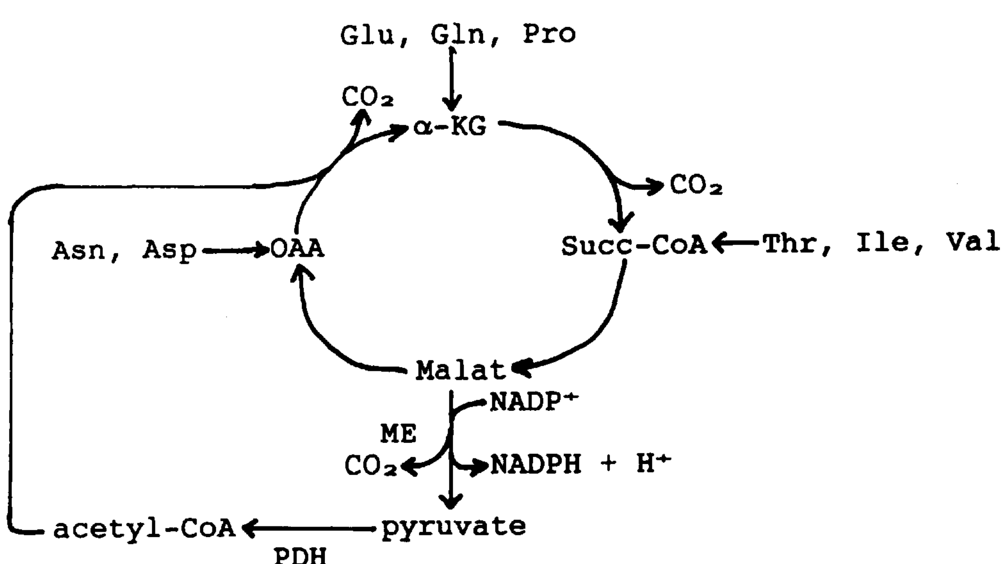

FIG. 32-4. Functions of malic enzyme in amino acid metabolism. The degradation of some amino acids generates members of the Krebs cycle. In order for the carbon skeleton to be consumed further, some constituent of the cycle has to be withdrawn. The most likely candidate is malate that can be degraded to pyruvate in a reaction catalyzed by malic enzyme (ME, EC 1.1.1.40). Pyruvate in turn can be taken care of by pyruvate dehydrogenase (*PDH*). The acetyl moiety of the acetyl-CoA formed is metabolized to CO_2 and H_2O in the Krebs cycle. *OAA*, oxaloacetate; *α-KG*, α-ketoglutarate; *Succ-CoA*, succinyl–CoA.

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33 Glia-specific enzyme systems

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Enzymes form the basis of every cellular function, and particular pathways require particular enzyme systems. Accordingly, glia-specific functions may be reflected in glia-specific enzyme systems. However, it should be stated clearly that there are no glia-specific enzymes that do not occur in cells outside the nervous system, however little activity they may exhibit. Therefore, in this chapter the term "glia-specific" will refer to (1) enzymes that are found exclusively in glial cells rather than neurons within the nervous system, (2) enzymes that are highly enriched in glial cells compared with neurons, and (3) enzymes that exist in glia-specific isoforms. All three criteria can also be applied to particular glial populations and even subpopulations determined, for example, by regional heterogeneity.

With the restrictions given above, what are the means of identifying a glia-specific enzyme? Measurement of enzyme activity is only useful in pure cell populations, for example, glioma cell lines, whereas the results derived from brain slices or mixed glial primary cultures (although usually devoid of neurons) are not unequivocal. However, the introduction of pure cultures of a particular glial cell type (e.g., Besnard et al., 1989; Juurlink and Hertz, 1991; Wiesinger et al., 1991) and the improvement of enzyme histochemical methodology (Kugler, 1988, 1991a) will lead to new insights into glial enzymology.

Thus far, the only unequivocal way to assign an enzyme to a particular cell type is the proper use of immunological methods. However, a careful choice of cell markers and a thorough characterization of the antiserum antibody used are mandatory. In particular, monospecificity of the antibody and lack of cross-reactivity with closely related (iso)forms of the respective antigen have to be proven. More recently, *in situ* hybridization techniques have rendered possible the study of the synthesis of particular mRNAs with subcellular resolution. A critical evaluation of the results of enzyme histochemistry, immunocytochemistry and/or *in situ* hybridization may give strong indications of the differential expression of enzyme activity or protein in heterogeneous cell populations.

Particular care has to be taken to prove the absence of an enzyme from a given cell type. A number of enzymes have been assigned to a glial cell population in the first place but then have been demonstrated to exist in other cell types as well (see the following section). Neuronal localization of a few enzymes later shown to be glia-specific probably resulted from the improper use of immunological methods (see the following section). Notwithstanding, a variety of enzymes appear to be exclusively expressed or at least highly enriched in particular glial cells (see the section *Enzymes Specific for One Glial Cell Population*). Yet there are some unresolved controversies regarding enzyme localization (see the section *Points of Controversy*).

ENZYMES PRESENT IN MORE THAN ONE GLIAL CELL TYPE

Within the nervous system, a number of enzymes are found in more than one glial cell type but not in neurons. Glutamine synthetase and carbonic anhydrase were taken as enzymatic markers for astrocytes and oligodendrocytes, respectively, but more recently were shown to exist in other glial cell populations as well. Glycogen phosphorylase, cytosolic malic enzyme, and the enzymes of the sorbitol pathway are indicators of specific metabolic capacities of glial cells that are generally lacking in neurons.

Glutamine Synthetase

Glutamine synthetase (GS) [L-glutamate:ammonia ligase (adenosine diphosphate); EC 6.3.1.2] catalyzes the conversion of glutamate to glutamine in the presence of adenosine triphosphate (ATP) and ammonia and, therefore, is a pivotal enzyme in nitrogen metabolism (see Chapter 63, this volume). In the central nervous system (CNS), the enzyme has the dual function of (1) the disposal of the neurotoxic substances glutamate and ammonia and (2) providing glutamine as a substrate for neuronal glutaminase as well as for further utilization in protein synthesis and oxidative metabolism. GS modulates not only the activity of

glutamatergic neurons but also plays a role in the metabolism of the inhibitory transmitter gamma-aminobutyric acid the transamination of which furnishes, as one of the products, glutamate as well. A compartmentation of GS (glial cells) and glutaminase (neurons) with cycling of the respective substrates between the two cell types has been proposed (for review see Kvamme, 1983). In accordance with the latter hypothesis GS has been detected in glial cells of the CNS only, although the idea of an exclusive localization in astrocytes has to be abandoned. Molecular and catalytic properties of the liver enzyme, which appears to be identical with the enzyme isolated from brain, have been reviewed (Meister, 1985).

For a long time GS was considered as a marker enzyme of astroglial cells. GS has been localized in astrocytes in immunocytochemical electron microscopic studies on ultrathin sections of adult rat brain. No positive staining was observed in neurons, oligodendrocytes, or microglial cells (Norenberg and Martinez-Hernandez, 1979). Human GS immunoreactivity was also found in astrocytic cytoplasm and endfeet with an antiserum that appeared to be monospecific (Yamamoto et al., 1987). Accordingly, in the retina GS is localized exclusively in Müller glia cells (Linser and Moscona, 1979).

These findings had been corroborated by investigations in primary cultures derived from neonatal mouse brain. These cultures are highly enriched in astroglial cells many of which could be stained with antiserum against pig brain GS (Hallermayer et al., 1981). Treatment of the cultures with the glucocorticoid dexamethasone elevated GS specific activity about sixfold and increased strongly the number of cells with positive GS immunoreactivity (Hallermayer et al., 1981). Less pronounced effects of a glucocorticoid were seen when the cultures were grown in the presence of 20% horse serum instead of 10% fetal calf serum (Juurlink et al., 1981), presumably because of the already higher concentration of glucocorticoids in the horse serum. The concentration of astroglial translatable GS-mRNA also increased in the presence of glucocorticoids (Khelil et al., 1990). GS activity in cultured astrocytes has been demonstrated to be regulated by hormones other than glucocorticoids and growth factors (triiodothyronine, insulin, fibroblast growth factor, epidermal growth factor) (for references, see Fressinaud et al., 1991) and by the presence of neurons (Wu et al., 1988; Mearow et al., 1990). Particularly high GS activity was found in highly enriched type-2 astrocyte cultures (Juurlink and Hertz, 1991).

However, recently it became clear that GS is not confined to astroglial cells but can also be found in oligodendrocytes *in vitro* as well as *in vivo*. This was shown for the first time in oligodendrocyte-enriched cultures from 7-day-old rat brain in which GS activity was markedly lowered when the number of galactocerebroside-positive cells was decreased by complement-mediated lysis (Warringa et al., 1988). In addition, cellular coexistence of galactocerebroside and GS was demonstrated by immunocytochemical double-labeling experiments (Warringa et al., 1988). Subsequently, the presence of GS was demonstrated in oligodendroglial cells in heterogeneous mouse glial primary cultures (Wiesinger et al., 1991) and in cultured rat oligodendrocytes (Fressinaud et al., 1991) by immunocytochemistry. Pure oligodendrocytes also exhibited a pronounced regulation of GS by hormones (Fressinaud et al., 1991). Very recently, GS immunoreactivity as well as GS mRNA have been found in cultured ependymal cells (Graff et al., 1993).

Glutamine synthetase immunoreactivity has been reported *in vivo* in perineuronal gray matter oligodendrocytes of the cerebral cortex, cerebellum, brainstem, and spinal cord of the adult cat (D'Amelio et al., 1990). GS-immunoreactive oligodendrocytes exist in the white matter of rat and bovine brain as was confirmed on oligodendrocytes isolated from these species (Tansey et al., 1991). Electron microscopic evidence for the presence of GS in oligodendrocytes of mouse brain was obtained by Miyake and Kitamura (1992).

Glial GS may be involved in pathologic states of the brain. For example, GS appears to be impaired in ferric chloride-induced epileptic foci, which implicates improper regulation of the neuronal environment in seizure regulation (Tiffany-Castiglioni et al., 1989). The finding of increased GS immunoreactivity and enzymatic activity following cerebral ischemia (Petito et al., 1992) underlines the importance of glial cells in normalizing extracellular glutamate concentrations and thereby protecting brain from the neurotoxic effects of this amino acid.

In summary, one can conclude that GS should not be used as an immunological marker for astroglial cells without additional evidence for the nature of the GS-positive cells. Therefore, GS as an indicator of the phenotype of C6 glioma cells (e.g., Kentroti et al., 1991) may also be regarded with caution. However, absence of GS from neurons and presence in more than one population of glial cells underlines the importance of extraneuronal glutamine synthesis from glutamate and validates the proposed function of GS in the glutamate-glutamine-cycle. In addition, the results may indicate that perineuronal oligodendrocytes functionally resemble more astrocytes than myelinating oligodendrocytes (D'Amelio et al., 1990).

Glycogen Phosphorylase

Glycogen phosphorylase (GP) [α-1,4-D-glucan: orthophosphate D-glucosyltransferase; EC 2.4.1.1] catalyzes the formation of glucose-1-phosphate from the storage polysaccharide glycogen. Three homodimeric isozymes predominantly located in liver, muscle, and brain have been described. The brain isoform also occurs in fetal tissues, including liver and muscle (for references, see Reinhart et al., 1990). The brain isoform was purified from bovine brain and shown to have a subunit molecular mass of 97 kD (Reinhart et al., 1990).

Attempts to localize GP in cells of neural tissue with immunocytochemical methods had yielded ambiguous results probably due to the fact that the antibodies produced against the liver or muscle isoform reacted only poorly or not at all with the other isoforms. With the monoclonal antibodies raised against purified bovine brain GP no immunoreactivity could be detected in neurons in rat neuron-rich primary cultures (Reinhart et al., 1990). Examination of rat astroglia-rich primary cultures revealed a colocalization of GP with glial fibrillary acidic protein (GFAP) in many cells. GP immunoreactivity was also seen in GFAP-negative cells, which tentatively were identified as ependymal cells (Reinhart et al., 1990).

The suggestion derived from this study that GP is localized predominantly in glial (astroglial and ependymal) cells was substantiated by investigations with the same antibody in formaldehyde-fixed, paraffin-embedded slices from adult rat brain (Pfeiffer et al., 1990). GP immunoreactivity was found in cells, which, by morphological criteria, were classified as astrocytes, and in the ependymal cells of the ventricles (Pfeiffer et al., 1990). Immunofluorescence double-labeling and immunoenzyme double-staining methods finally demonstrated in these brain sections a strict coexpression of GP and GFAP as well as S-100 protein in cells of the cerebellum and hippocampus (Pfeiffer et al., 1992). In the cerebellum, both GP and GFAP were found in the fibrous astrocytes of the white matter, in astrocytes of the granule layer, and in the radial fibers of the Bergmann glia cells. Similar coincidence was shown for GP and S-100 protein in the same cell types. In the hippocampus, all GFAP-positive astrocytes exhibited a high level of GP immunoreactivity, with GP staining being particularly intense in the fine fibers of protoplasmic astrocytes surrounding neurons. In GFAP-negative ependymal cells, GP was colocalized with S-100 protein.

Immunohistochemical studies in human brain sections with antiserum against human brain-GP also revealed that the enzyme is present in astrocytes (Kato et al., 1989). Weak immunoreactivity was found in some neurons of the cerebral cortex and in the Golgi cells of the cerebellar cortex, whereas Purkinje and granule cells were devoid of GP (Kato et al., 1989). These findings are in large part in accordance with the results of an investigation that used antiserum against the epitope corresponding to the terminal 12 amino acids of the human brain GP on sections of macaque and rat cerebrum and cerebellum (Ignacio et al., 1990). The highest level of immunostaining was found in many of the fibrous astrocytes in white matter, which had processes ending on blood vessels. In addition, immunostaining was found in protoplasmic astrocytes of cerebral gray matter. No immunostaining was seen in Bergmann glia cells (Ignacio et al., 1990).

With the immunochemical methods described above GP was clearly demonstrated to be a glial enzyme located in astrocytes as well as in ependymal cells. The function of GP in these cells still has to be elucidated. Breakdown of glycogen in the ependymal cells may furnish the energy required for continuous beating of the cilia during states of reduced glucose supply, but release of glucose (or an equivalent compound) to the cerebrospinal fluid has also to be considered. The finding of GP being primarily localized in astrocytes points to the crucial role of this cell type in the energy metabolism of the brain (for a thorough discussion, see Chapter 32).

Malic Enzyme

Malic enzyme (ME) [L-malate:$NADP^+$ oxidoreductase (decarboxylating); EC 1.1.1.40] catalyzes the reversible formation of pyruvate, CO_2, and NADPH from malate and $NADP^+$ in the presence of the essential cofactors Mg^{2+} or Mn^{2+}. From rat and bovine brain two forms of the enzyme have been separated, a mitochondrial form and a cytosolic form, which can be distinguished by kinetic or electrophoretic properties. Cytosolic ME (cME) was purified from bovine brain and shown to be a homotetramer with a subunit molecular mass of 60 kD (Kurz et al., 1993). A previous immunocytochemical investigation had reported cME to be present in glial cells as well as in neurons of the cerebellum (Martínez-Rodríguez et al., 1989). However, this study was hampered by the fact that neither monospecificity nor cross-reactivity with cME from brain of the antiserum prepared against cME from chicken liver had been demonstrated. This is of particular importance, since Kurz et al. (1993) showed that rabbit polyclonal antiserum against cME from bovine brain reacted only weakly with cME purified from chicken liver. In contrast, a monoclonal anti-

body against cME from bovine brain was well suited for immunocytochemical investigations (Kurz et al., 1993). In rat glial primary cultures, cME immunoreactivity was found in GFAP-positive cells (astroglial cells), GFAP-negative cells (presumably ependymal cells) as well as in GFAP-negative, myelin basic protein- or galacto cerebroside-positive cells (oligodendroglial cells). The latter cell population exhibited particularly strong immunoreactivity, which may reflect a role of cME in NADPH production required for the lipid synthesis in myelinating cells. cME immunoreactivity was found neither in neurons in rat neuron-rich primary cultures (Kurz et al., 1993) nor in Purkinje cells and other neurons in adult rat brain slices. In these brain sections, however, cME could be detected in astrocytes and oligodendrocytes in the hippocampus and cerebellar white matter, and in Bergmann glia cells of the cerebellar cortex (G. Kurz, H. Wiesinger, and B. Hamprecht, unpublished observations).

In conclusion, cME can be considered a glia-specific enzyme present in astrocytes, oligodendrocytes, and ependymal cells. Since cME may be implicated in functions as diverse as glutamate metabolism or reduction of oxidized glutathione (Kurz et al., 1993), its presence in glial cells is a further example of the restriction of important metabolic processes to these cells within the CNS.

Carbonic Anhydrase

Carbonic anhydrase (CA) [carbonate hydrolyase; EC 4.2.1.1] catalyzes the reversible hydration of CO_2 yielding HCO_3^- and a proton. The CA gene family contains seven genes, each of which encodes an isoenzyme (CA I–CA VII) with distinct kinetic and, as far as determined, immunological properties (Tashian, 1989). To date, only CA II, with a molecular mass of 31 kD, has been detected in CNS parenchyma, whereas membrane-associated CA IV is expressed exclusively in brain capillary endothelial cells (Ghandour et al., 1992).

Increasing amounts of CA II appear in the particulate fraction of brain homogenates during postnatal development, and in the adult rodent as much as 50% of CA II was found to be bound to the plasma membrane, including myelin (Ghandour et al., 1980). Association of CA II with myelin was deduced from these studies, and, accordingly, CA enzyme activity and immunoreactivity were found in oligodendrocytes (Ghandour et al., 1989). *In situ* hybridization analysis detected the transcript for CA II only in cultured mouse oligodendrocytes (Ghandour and Skoff, 1991).

Nevertheless, CA was also detected in astroglial cells. Primary cultures derived from neonatal rat cerebral hemispheres exhibited CA activity, which could not be accounted for solely by the small percentage of oligodendrocytes in this astroglia-rich culture system (Kimelberg et al., 1982). In immunocytochemical experiments, the weak staining for CA observed in the cultured astroglial cells could be increased to an intensity equaling that of the oligodendrocytes after the cells had been treated with dibutyryl cAMP (Kimelberg et al., 1982). In the rodent brain, CA is expressed in some astrocytes of the gray matter, as demonstrated in double-labeling experiments with anti-CA antiserum and antibodies against GS and, more convincingly, GFAP (Cammer and Tansey, 1988). Enhanced expression of CA in astrocytes was also observed as a response to injury (Cammer et al., 1989b). Finally, CA immunoreactivity was found in vimentin-positive precursor cells in the rat forebrain as early as the first postnatal day (Cammer and Zhang, 1992).

In summary, it is clear that CA II is a glia-specific enzyme in the CNS, and it appears to be highly enriched in oligodendrocytes. The enzyme is lacking in neurons, although a CA-related peptide with unknown function has been detected in Purkinje cells (Kato, 1990). Besides its role in ion and pH homeostasis (see Chapter 14, this volume), consistent with a glial localization of CA in areas with a high rate of CO_2 production (Sapirstein, 1983), CA in the glial cells of the CNS may have additional functions. Double immunofluorescence staining showed CA and carbamoylphosphate synthetase II in the same astrocytes of rodent brain, which suggests that CA, together with GS detected in the same cells, could furnish the substrates bicarbonate and glutamine for pyrimidine synthesis (Cammer, 1991; see also the subsection *Multifunctional Protein CAD*). A similar colocalization of CA, acetyl-CoA carboxylase, and fatty acid synthetase may hint at a role of CA in another biosynthetic process (Cammer, 1991). An anaplerotic role of CA had already been suggested on the grounds of an astroglial coexpression of CA and pyruvate carboxylase that uses bicarbonate as substrate (Sapirstein, 1983).

Aldose Reductase and Sorbitol Dehydrogenase

Aldose reductase (AR) [alditol:NADP$^+$ 1-oxidoreductase; EC 1.1.1.21] and sorbitol dehydrogenase (SDH) [L-iditol:NAD$^+$ oxidoreductase; EC 1.1.1.14] constitute the so-called polyol (sorbitol) pathway, the activity of which results in the conversion of a variety of aldoses to the corresponding ketoses through a polyol intermediate. AR purified from hu-

man brain is a monomer with a molecular mass of 38 kD (Wermuth et al., 1982), whereas each subunit of homotetrameric SDH purified from bovine brain has a molecular mass of 39 kD and contains one zinc atom (Wiesinger and Hamprecht, 1989). Although a physiological function of the widely distributed sorbitol pathway is not known, accumulation of pathway substrates under hyperglycemic conditions has been implicated in the etiology of diabetic complications (Cohen, 1987). Since peripheral neuropathy is a major sequelae of long-lasting hyperglycemia, distribution of the sorbitol pathway enzymes in nervous tissue is of particular interest.

Early biochemical studies using Wallerian degeneration experiments on sciatic nerve had suggested that AR is confined to the Schwann cell, whereas SDH is localized in the axon (Gabbay and O'Sullivan, 1968). Later on, studies with antiserum against AR purified from seminal vesicles confirmed that AR is localized in the Schwann cell cytoplasm of rat sciatic and cranial nerves, and that axons are devoid of AR immunoreactivity (Ludvigson and Sorenson, 1980). Expression of AR as a function of differentiated or mature Schwann cells was also demonstrated recently (Wong et al., 1992). So far the restriction of SDH to the axon was not corroborated by immunocytochemical evidence. However, during studies on polyol pathway enzymes in cell cultures derived from rat central nervous system, AR as well as SDH enzyme activities were detected only in glial cells but not in neurons (Wiesinger et al., 1990). Moreover, with antiserum raised against purified bovine brain SDH, immunoreactivity was found in astroglial as well as in oligodendroglial cells of heterogeneous glial cultures derived from mouse brain (H. Wiesinger, unpublished observations). This finding is of interest, since the SDH-positive oligodendrocytes disappear from the culture after replacement of glucose in the growth medium by sorbitol, which is the basis of a convenient method to generate pure astroglia cultures of high cell density (Wiesinger et al., 1991). Although the functional significance of the restriction of AR and SDH to glial cells in the central and at least of AR to the Schwann cells in the peripheral nervous system is far from being clear, these findings demonstrate once more the metabolic versatility of glial cells.

ENZYMES SPECIFIC FOR ONE GLIAL CELL POPULATION

Oligodendrocytes and Schwann Cells

Since the major function of oligodendrocytes in the CNS as well as of Schwann cells in the peripheral nervous system (PNS) is the formation and maintenance of the myelin sheath, myelin-associated enzymes have to be considered as being specific to this class of neural cells. In addition, glycerol-3-phosphate dehydrogenase may be an enzymatic marker for oligodendrocytes.

Myelin-Specific Enzymes. Myelin-associated enzymes together with criteria defining myelin enzymes have been discussed expertly (Lees and Sapirstein, 1983; Vogel and Thompson, 1988; see Section VII, this volume). Therefore, only some recent findings will be presented in the following sections. Exclusively located in myelin, and therefore glia-specific within the nervous system, are only a few enzymes involved in lipid metabolism together with 2′,3′-cyclic nucleotide 3′-phosphodiesterase. Carbonic anhydrase has been localized in astroglial cells as well and is discussed in the preceding section *Carbonic Anhydrase.*

2′,3′-Cyclic nucleotide 3′-phosphodiesterase. 2′,3′-Cyclic nucleotide 3′-phosphodiesterase (CNP) [EC 3.1.4.37] hydrolyzes 2′,3′-cyclic nucleoside monophosphates with the release of 2′-phosphates. The former compounds are found only as intermediates in the reaction catalyzed by ribonuclease and may not represent the physiological substrates. Therefore, also the proper function of CNP remains an enigma. In a number of species, the enzyme is expressed in two isoforms with molecular masses of 46 and 48 kD (data from rat brain), which apparently are identical to the Wolfgram proteins and are the products of separate, alternatively spliced mRNAs (Bernier et al., 1987; Kurihara et al., 1990). A rabbit autoantibody specific for the 46 kD form was detected recently and might prove useful for distinguishing between the two CNP isoforms (Möller et al., 1992).

CNP has been detected in myelin of the CNS and PNS and in oligodendrocytes and Schwann cells that synthesize central and peripheral myelin, respectively. To a far lesser degree, CNP activity can also be found in nonneural tissues (for detailed discussion see Lees and Sapirstein, 1983, and Vogel and Thompson, 1988). CNP activity can be induced by cyclic AMP in cultured oligodendrocytes and C6 glioma cells, and it was shown, at least for the glioma cells, that the rate of synthesis of CNP is increased by cyclic AMP (McMorris et al., 1985). Other glial cell lines express CNP as well (Almazan and McKay, 1992). CNP mRNA was found exclusively in oligodendrocytes in the CNS (Trapp et al., 1988; Vogel et al., 1988). CNP may be a marker for oligodendrocyte differentiation, since it is enriched in a myelin fraction that is a transitional region between the oligodendroglial plasma membrane and mature multilamellar myelin (Trapp et al., 1988; Gil-

lespie et al., 1989). Association of CNP with the cytoskeleton in cultured oligodendrocytes was also reported (Dyer and Benjamins, 1989). Regulation of CNP gene expression in the PNS under normal and pathophysiological conditions was described recently (LeBlanc et al., 1992).

Cholesterol ester hydrolase. Of several enzymes involved in the modification of myelin lipids, a particular isoform of cholesterol ester hydrolase (CEH) [EC 3.1.1.13] appears to be enriched in myelin (Eto and Suzuki, 1973). The enzyme catalyzes the hydrolytic cleavage of cholesterol esters, yielding cholesterol and the free fatty acids. The myelin enzyme can be distinguished from a microsomal isoform by its pH optimum of activity (7.2 vs 6.0 of the microsomal enzyme) (Eto and Suzuki, 1973). Increase in CEH activity coincides with the period of active myelination (Lees and Sapirstein, 1983), and a physiological role for this enzyme in synthesis and maintenance of myelin has been inferred (Ghosh and Grogan, 1990). The role of CEH in experimental allergic encephalomyelitis has been discussed recently (Ghosh and Grogan, 1991).

UDP-galactose:ceramide galactosyltransferase. UDP-galactose:ceramide galactosyltransferase (CGalT) [EC 2.4.1.45] catalyzes the final step in the biosynthesis of galactosyl ceramide (cerebroside) by transferring a galactose moiety from the sugar nucleotide to ceramide. Since cerebroside is a lipid that occurs almost exclusively in myelin, CGalT may be considered a myelin-specific enzyme (Lees and Sapirstein, 1983). With immunocytochemical methods CGalT was localized in the outermost and innermost lamellae of the myelin sheath (Roussel et al., 1987), and expression of CGalT was demonstrated in cultured oligodendrocytes in a development-dependent manner (Nescovic et al., 1988). Levels of CGalT activity were comparable in homogenates from oligodendrocytes and the light myelin fractions (Saito et al., 1992), whereas CGalT activity was enriched 20-fold in purified rat oligodendrocytes as compared with brain homogenates, which led to the proposal that CGalT may be a proper enzyme marker for mature oligodendrocytes (Lubetzki et al., 1991).

Miscellaneous myelin-associated enzymes. Recently, a variety of enzymes have been described that appear to be enriched in oligodendrocytes and to be essential for the proper synthesis and function of myelin.

The RLM_6 form of cytochrome P-450 was localized immunocytochemically in oligodendrocytes of rat brain. A contribution of the enzyme to the energy supply in myelin through the utilization of ketone bodies was discussed (Cammer et al., 1991).

With *in situ* hybridization, the 80 kD isoform of diacylglycerol kinase was found to be expressed in oligodendrocytes of rat brain, and a role in myelin maturation was assigned to this enzyme (Goto et al., 1992).

The Y_p-subunit isoform of dimeric glutathione-*S*-transferase [EC 2.5.1.18] (see also the section *Isoforms of Miscellaneous Enzymes* below) was found exclusively in oligodendrocytes. The enzyme was suggested to remove toxic substances from the vicinity of the myelin sheath (Cammer et al., 1989a).

Plasma membrane-bound carboxypeptidase M [EC 3.4.17.12], which cleaves C-terminal basic amino acids of peptides was found closely associated with myelin and myelin-forming cells in the CNS as well as the PNS (Nagae et al., 1992).

Similarly, in the PNS, endopeptidase-24.11 [EC 3.4.24.11], an enzyme initiating the degradation of several peptide hormones, was found exclusively on Schwann cell membranes (Barnes et al., 1991).

Finally, glycerophosphorylcholine phosphocholine phosphodiesterase [EC 3.1.4.38] was found associated with purified rat brain myelin and in pure cultures of oligodendrocytes, but not in cultured neurons or astrocytes (Yuan et al., 1992).

Glycerol-3-Phosphate Dehydrogenase. L-Glycerol-3-phosphate dehydrogenase (GPDH) [L-glycerol-3-phosphate:NAD^+ oxidoreductase; EC 1.1.1.8] reduces dihydroxyacetone phosphate with the help of NADH to yield α-glycerol phosphate, and thus is part of the cyclic metabolic process which transfers reducing equivalents from the cytosol to the mitochondria. In paraformaldehyde-fixed sections of adult rat brain the enzyme was detected with immunoperoxidase staining procedures in oligodendrocytes exclusively (Leveille et al., 1980). Also in these cells, GPDH can be specifically induced by glucocorticoid hormones (Leveille et al., 1980). These findings may reflect the need of phospholipid synthesis in the myelinating cells, whereas a preference for the glycerol phosphate shuttle over the malate-aspartate shuttle (which yields an additional mole of ATP per mole of NADH) in the oligodendrocytes cannot be explained easily.

Astrocytes

In addition to the enzymes that astrocytes share with other glial cell types (see the section *Enzymes Present in More Than One Glial Cell Type* above), a variety of enzymes has been found in the CNS in astrocytes exclusively. In particular, enzymes catalyzing reactions involved in CO_2 fixation and the biosynthesis of the neurotransmitter glutamate, in the catabolism of neurotransmitters, or in detoxification of xenobiotics appear to be constituents of

astrocytes or are expressed in these cells in specific isoforms.

Pyruvate Carboxylase. Pyruvate carboxylase (PC) [pyruvate:CO_2 ligase (ADP); EC 6.4.1.1] is located in the mitochondrial matrix and catalyzes the formation of oxaloacetate from pyruvate and CO_2, which leads to a net synthesis of tricarboxylic acid cycle intermediates. Studies with antiserum against rat liver PC localized the enzyme in sections of adult mouse cerebellum in astrocytes exclusively (Shank et al., 1985). This finding is in accordance with an earlier report that PC enzymatic activity was present only in primary cultures of astrocytes but was detectable neither in cultured cerebral cortex neurons nor in cerebellar granule cells (Yu et al., 1983). These results implicate that the major CO_2-fixing cells of the brain are indeed the astrocytes being able to replenish constituents of the tricarboxylic acid cycle, which had been removed as precursors for the neurotransmitters glutamate and gamma-aminobutyric acid.

Glutamate Dehydrogenase. In the brain, mitochondrial NADH-dependent glutamate dehydrogenase (GDH) [L-glutamate:NAD^+ oxidoreductase; EC 1.4.1.2] catalyzes the reductive formation of glutamate from α-ketoglutarate and ammonia. Catalytic enzyme histochemistry was used to demonstrate that GDH is enriched in Bergmann glia cells and astrocytes in the rat hippocampus and cerebellar cortex (Kugler, 1991b; Würdig and Kugler, 1991). A similar preferential localization of GDH in astrocytes was suggested on the grounds of immunocytochemical investigations (Aoki et al., 1987; Wenthold et al., 1987). Therefore, α-ketoglutarate provided by the tricarboxylic acid cycle can be directly converted to glutamate within the astrocytes, which, thanks to this reaction, also have an additional means for the detoxification of ammonia.

1-Pyrroline-5-Carboxylate Dehydrogenase. 1-Pyrroline-5-carboxylate dehydrogenase [EC 1.5.1.12] catalyzes the NAD^+-dependent oxidation of 1-pyrroline-5-carboxylate (derived either from ornithine or proline) to glutamate. With histochemical staining methods, the enzyme was detected in Bergmann glia cells in the cerebellum and astrocytes in the hippocampus of the adult rat, but not in neurons (Thompson et al., 1985). Therefore, only glial cells possess this alternative route of glutamate production (Thompson et al., 1985).

Multifunctional Protein CAD. A multifunctional polypeptide named "CAD" exhibits the enzymatic activities for carbamoyl phosphate synthetase II, aspartate transcarbamoylase, and dihydroorotase and thus furnishes, from glutamine, HCO_3^-, and ATP, dihydroorotate, the precursor of pyrimidines. With an antiserum against hamster CAD, the polypeptide was localized exclusively in astrocytes of normal hamster and rat brains and in the gliotic brains of myelin-deficient mutant rats (Cammer and Downing, 1991). In hamster brain, CAD was also found in Bergmann glia cells and tanycytes. Therefore, with the additional help of glutamine synthetase and carbonic anhydrase, which are also localized in astrocytes (see the sections *Glutamine Synthetase* and *Carbonic Anhydrase* above), these cells are able to perform the *de novo* synthesis of pyrimidines, which might be required in proliferating cells, besides the predominant salvage pathway.

Glycine Cleavage System. The glycine cleavage system, also called glycine synthase (GCS) [EC 2.1.2.10] catalyzes the degradation of glycine to yield methylenetetrahydrofolate, CO_2, and ammonia. It is located on the outer mitochondrial matrix, consists of four separate enzymes, and constitutes the major catabolic pathway for glycine in vertebrates (for references, see Sato et al., 1991). With antiserum against the P protein of GCS the multienzyme complex was detected in rat brain only in astrocytes and Bergmann glia cells, and in the retina only in Müller cells (Sato et al., 1991).

Isoforms of Miscellaneous Enzymes. The Y_b subunit of glutathione-*S*-transferase [EC 2.5.1.18] (see also the subsection *Miscellaneous Myelin-Associated Enzymes* above) was primarily found in astrocytes (and ependymal cells) in rat brain and appears to be absent from oligodendrocytes and neurons (Cammer et al., 1989a). In view of the anatomical position of astrocytes in the brain, these cells, therefore, may play a pivotal role in the brain's defense against toxic substances. A preferential astroglial localization of protein kinase C isoform III in human brain was postulated on the grounds of immunohistochemical studies (Todo et al., 1990). Similarly, phospholipase-δ appears to be the isoform that is enriched in astrocytes (Mizuguchi et al., 1991). Finally, astrocytes express predominantly the α_1-subunit of Na^+,K^+-ATPase that has low affinity for ouabain (Atterwill et al., 1984).

Microglial Cells

Only scarce data are available on enzymatic activities associated with microglial cells. Ectoenzymes are enzymes located on the plasma membrane, their active site being oriented toward the external surface

of the cell. Two such enzymes may be characteristic for microglial cells.

Ecto-NAD$^+$ glycohydrolase [EC 3.2.2.6] catalyzes the hydrolysis of pyridine dinucleotides present in the extracellular environment. The enzyme is a marker of phagocytic cells, since it is highly expressed in peritoneal macrophages or Kupffer cells of the liver. Ecto-NAD$^+$ glycohydrolase activity was also found on the surface of cultured brain microglial cells, and its activity increased with time in correlation with the increased phagocytic capacity of the cells during time in culture. Therefore, this enzyme was considered a marker of the functional state of cultured microglial cells (Bocchini et al., 1988).

Another ectoenzyme, 5′-nucleotidase [5′-ribonucleotide phosphohydrolase; EC 3.1.3.5] catalyzes the hydrolysis of 5′-phosphate esters of ribo- and deoxyribonucleotides. Besides bacteria and plants, the enzyme is also widely distributed in vertebrate tissues (Zimmermann, 1992). In the PNS the enzyme is found associated with the cell membrane of Schwann cells (Grondal et al., 1988; Nacimiento et al., 1991) and, in the CNS, on a variety of glial elements like Bergmann glia, astrocyte endfeet, and oligodendrocytes (Schoen et al., 1987). Therefore, 5′-nucleotidase appears not to be specific for a particular glial cell type and may be restricted to the glial cell population as a whole, since it was only occasionally located on neuronal surfaces in adult nervous tissue (e.g., Cunha et al., 1992; for discussion, Nacimiento et al., 1991; Zimmermann, 1992). However, 5′-nucleotidase immunoreactivity was found to be restricted to perineural microglia, at least as far as experiments were performed with the monoclonal antibody 5N4-2 (Schoen et al., 1992). This antibody may distinguish between structural variants of 5′-nucleotidases, and a function of the latter protein as an adhesion molecule on microglial cells participating in regeneration processes after axotomy has been discussed (Schoen et al., 1992).

POINTS OF CONTROVERSY

Controversial literature on cellular location exists on a few enzymes that are expressed in multiple forms or as genetically distinct isozymes. The most prominent examples are discussed. In addition, most recent literature sheds new light on the localization of cysteine sulfinate decarboxylase, the pivotal enzyme of taurine biosynthesis.

Hexokinase

Hexokinase (HK) [ATP:D-hexose 6-phosphotransferase; EC 2.7.1.1], the first enzyme of the glycolytic sequence, is of utmost importance in energy metabolism. Most of what is known about mammalian hexokinases is derived from studies on the brain enzyme, notably those done in the laboratory of J. E. Wilson. Brain HK is of isoform I type, and was cloned and sequenced recently (Schwab and Wilson, 1989). The enzyme is a monomer with a molecular mass of 100 kDa. HK exists as a soluble cytosolic form and a particulate one that is the same enzyme but is bound to the outer mitochondrial membrane with concomitant changes in the kinetic properties (Wilson, 1980). Based on regional differences in the activity of cytosolic HK, Bigl had already suggested a predominantly glial localization of the soluble form of HK (Bigl et al., 1971). Studies in cultured astrocytes derived from neonatal rat brain led to the conclusion that about 80% of the astroglial HK activity is due to the soluble form, and only 20% of astroglial HK is bound to mitochondria (Lusk et al., 1980). In agreement with Wilson's suggestion that predominance of cytosolic HK points to an important role of nonoxidative metabolism in a particular cell type (Wilson, 1980), astroglial energy metabolism may indeed be dominated by glycolysis (Lusk et al., 1980).

However, the studies described above required the disruption of the cells for biochemical analysis, and the data have been questioned recently on the grounds of a study using three-dimensional confocal microscopy, a method that can analyze the subcellular distribution of immunoreactivity (Lynch et al., 1991). About 70% of HK in cultured astrocytes was found to be associated with mitochondria under basal metabolic conditions, and the amount of HK bound to mitochondria varied with the metabolic state of the cells (Lynch et al., 1991).

Creatine Kinase

Creatine kinase (CK) [ATP:creatine phosphotransferase; EC 2.7.3.2] catalyzes the reversible formation of phosphocreatine from creatine and ATP, and thus participates in energy storage and transport within the cell (Wallimann et al., 1992). Besides several cytosolic isoforms, also genetically distinct mitochondrial CKs exist (Wallimann et al., 1992). The brain possesses a particular cytosolic isoenzyme, CK-BB which has been localized by immunohistochemical methods with a specific antiserum in astrocytes in the white matter of human cerebrum (Thompson et al., 1980). Immunocytochemical experiments detected CK-BB also, particularly in Bergmann glia cell bodies of chick cerebellum (Scalabrini et al., 1989), and the phosphocreatine circuit model was applied

to astrocytes (Wallimann et al., 1992). However, CK activity was found in cultured astrocytes and oligodendrocytes as well as neurons (Manos et al., 1991), and CK-mRNA was detected in cultured astrocytes, oligodendrocytes, and neurons, although at a low level in the latter cell type (Molloy et al., 1992). It was concluded that ATP regeneration is an important process in all types of glial cells but may be restricted to a particular set of neurons involved in specialized metabolic functions (Molloy et al., 1992).

Cysteine Sulfinate Decarboxylase

Cysteine sulfinate decarboxylase (CSD) [EC 4.1.1.29] catalyzes the decarboxylation of cysteine sulfinic acid to yield aminoethane sulfinic acid (hypotaurine), the immediate precursor of aminoethane sulfonic acid (taurine). Although this amino acid is abundant in the CNS, its role is far from being elucidated. In accordance with the proposal that taurine might be a neurotransmitter or -modulator, in an early study CSD was localized immunocytochemically in the cerebellum in Purkinje cells and other types of neurons (Chan-Palay et al., 1982). However, the antiserum used may not have possessed the specificity required for unambiguous immunostaining (Almarghini et al., 1991). With monospecific antiserum against purified CSD the enzyme was detected in glial cells, astrocytes as well as oligodendrocytes, in the rat cerebellum. No labeling was found in Purkinje cells or the stellate cells of the molecular layer (Almarghini et al., 1991). This suggests that taurine is indeed synthesized only by glial cells, and the function of taurine in the CNS has to be reconsidered.

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34 Second-messenger systems

MARTHA L. SIMMONS AND SEAN MURPHY

As has been discussed in other chapters, glia have numerous cell-surface receptors (see Chapters 21 to 25, this volume). Responses to specific receptor activation in astrocytes are diverse, ranging from gross changes in morphology to glycogen metabolism and the release of neuroactive peptides and prostaglandins (see Murphy, 1993; see also Chapters 24 and 25). In contrast, receptors on microglia are only now beginning to be probed (Kettenmann et al., 1993). This chapter discusses some of the intracellular events that link receptors to the eventual responses of the glial cell. Such signal transduction can occur via receptors that are gated ion channels, receptors linked through G proteins to cyclases or phospholipases, or receptors with intrinsic enzyme activity (tyrosine kinase, guanylyl cyclase). For the sake of clarity, the focus here is on G protein-coupled receptors in astrocytes. Most studies to date have used primary cultures of astrocytes or astrocytoma-derived cell lines. General aspects of second-messenger systems will be presented first, followed by evidence for their expression and functional operation.

G PROTEIN-COUPLED RECEPTORS GENERATE A VARIETY OF SECOND MESSENGERS

Guanine nucleotide-binding proteins (G proteins) link some cell surface receptors with ion channels or enzyme systems (for reviews see Birnbaumer, 1990; Simon et al., 1991). These G proteins are heterotrimers comprising $\alpha\beta\gamma$ subunits, each of which exists as multiple isoforms. Upon interaction with an activated receptor, the subunits disassociate into an α subunit and a $\beta\gamma$ moiety, both of which are capable of activating an effector, although both are not necessarily active in any given receptor system. During this process the α subunit hydrolyzes guanosine triphosphate (GTP) to guanosine diphosphate (GDP), at which point it is again inactive. This system allows for amplification of a receptor signal, since each receptor can interact with more than one G protein, and each G protein can interact with more than one effector. The α subunits appear to be the most variable, with at least 15 isoforms identified. These can be specific for a given system (i.e., α_t, which links rhodopsin to cyclic guanosine monophosphate (GMP) phosphodiesterase in the visual system) or used by more than one receptor, as may be true for some of the α_i proteins. Some of these α subunits (α_s) are susceptible to ADP-ribosylation by cholera toxin, which causes a constitutive activation, while others (α_i) are ADP-ribosylated by pertussis toxin, which uncouples the G protein from associated receptors and hence prevents activation. Such toxins have proved useful tools for investigating the roles for various G proteins in a given system.

Phospholipases

Phospholipase C (PLC) hydrolyzes phospholipids to diacylglycerol (DAG) and the corresponding polar headgroup (see Figure 34-1). Of the inositol-specific PLCs the β, γ, and δ forms have been cloned (for review see Cockcroft and Thomas, 1992). PLCβ and PLCδ are generally linked to cell surface receptors via specific G proteins, while the γ form is regulated by receptor tyrosine kinases such as the platelet-derived growth factor receptor and the epidermal growth factor receptor.

The DAG formed by the hydrolysis of phospholipids activates protein kinase C, which then phosphorylates serine and threonine residues on target proteins. If the PLC acts upon phosphoinositide-4,5-bisphosphate (PIP_2), inositol-1,4,5-trisphosphate (IP_3) is concurrently released. IP_3 activates receptors located on organelles, such as the endoplasmic reticulum, causing the mobilization of calcium from these internal stores and raising intracellular free calcium $[Ca^{2+}]_i$. Both PKC activation and the rise in $[Ca^{2+}]_i$ are capable of modulating other second-messenger systems, as well as influencing more direct responses, such as Ca^{2+}/calmodulin activation of Ca^{2+}/calmodulin-dependent kinase II (CaM kinase II) and constitutive nitric oxide synthase, or phosphorylation of membrane receptors (see Figure 34-2).

In addition to PIP_2, a PLC can hydrolyze phosphatidylinositol (PI) or PIP leading to the production of IP or IP_2, and there is evidence that these reactions are more calcium-dependent than the PLC hydroly-

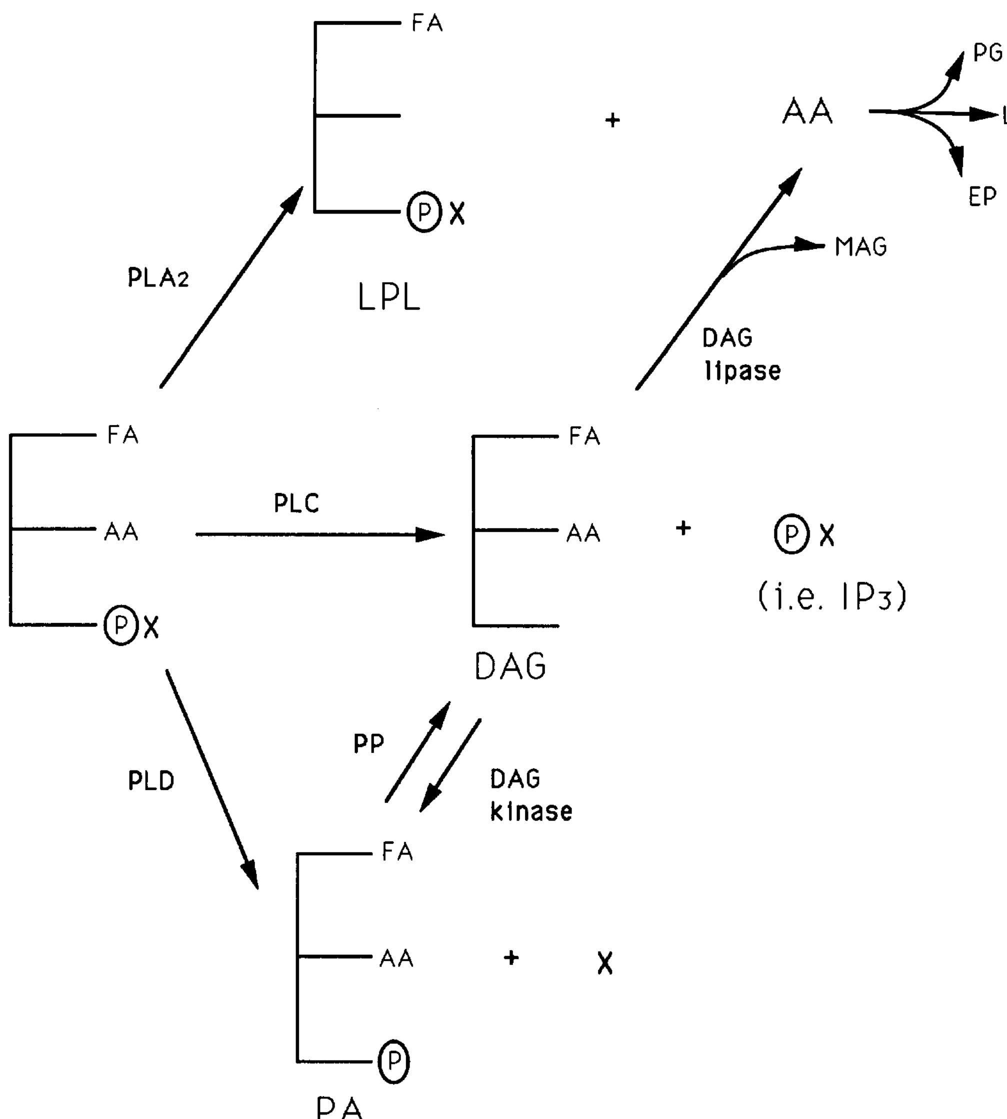

FIG. 34-1. Generation of second messengers by phospholipases. Phospholipase A_2 (*PLA_2*) cleaves arachidonic acid (*AA*) from the phospholipid leaving a lysophospholipid (*LPL*). AA can then be converted to prostaglandins (*PG*), leukotrienes (*LT*), or epoxyeicosatrienoic acids (*EP*). Actions of phospholipase C (*PLC*) results in the production of diacylglycerol (*DAG*) and a phosphorylated polar head group (e.g., inositol trisphosphate, IP_3). Phospholipase D cleaves off the polar head group (*X*) to form phosphatidic acid (*PA*). These products can be interchanged by the actions of phosphatidate phosphatase (*PP*), DAG kinase, and DAG lipase. See text for details.

sis of PIP_2 (Fain, 1990). The resulting inositol phosphates can also be interchanged by the actions of kinases and phosphatases, forming various isomers of IP_3 as well as IP_4 and IP_5. In some cases, this is simply a method of inactivation of the messenger, but there is evidence that IP_4, and possibly IP_5, may play a role in calcium signaling.

Phospholipase D (PLD) hydrolyzes phospholipids to yield phosphatidic acid and the corresponding base (for reviews see Shukla and Halenda, 1991, Thompson et al., 1991) (see Figure 34-1). Although the existence of the enzyme has long been recognized, only recently has it been implicated in signal transduction. Different forms of PLD may be activated by such compounds as carbachol, ATP, synthetic diacylglycerol, calcium ionophores, epidermal growth factor, protein kinase C, and vasopressin, some of these through the action of G proteins. Phosphatidic acid itself may have some second-messenger roles, or it can be further hydrolyzed to diacylglycerol by phosphatidate phosphatase. Protein kinase C activated via this method acts as a positive regulator of PLD, in contrast to the negative feedback on PLC, and may therefore provide a more long-term signal than can PLC (Shukla and Halenda, 1991). Conversely, diacylglycerol formed via PLC

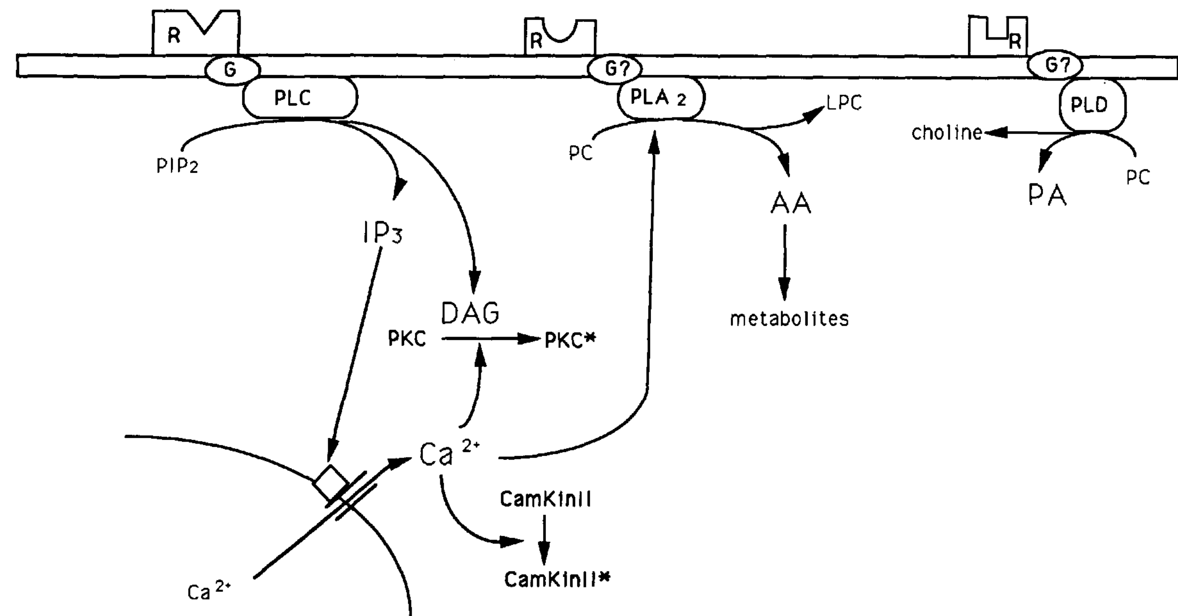

FIG. 34-2. Roles of phospholipases in intracellular signaling. Stimulation of G protein (*G*) coupled receptors (*R*) results in activation of phospholipases, which then generate a variety of second messengers. See text for details. *PKC*, protein kinase C; *PC*, phosphatidylcholine; *PIP_2*, phosphatidylinositol bisphosphate; *CamKinII*, calcium/calmodulin-dependent kinase II; *, activated kinase. For other abbreviations see legend to Figure 34-1.

can be phosphorylated by diacylglycerol kinase to form phosphatidic acid.

Phospholipase A_2 (PLA_2) hydrolyzes phospholipids to lysophospholipids and arachidonic acid (for review see Axelrod, 1990) (see Figure 34-1). The enzyme is calcium-sensitive and is activated by compounds such as angiotensin, α-adrenergic agonists, bradykinin, ATP, and arginine vasopressin—in some cases via the action of a G protein. In the visual system, the βγ subunits of the G protein activates PLA_2, but in other systems pertussis toxin-sensitive α subunits are the activators.

Arachidonic acid can also be produced by further reactions with the products of PLC and PLD. Diglyceride lipase can cleave arachidonic acid from PLC-produced diacylglycerol, while a PLA_2 can cleave arachidonic acid directly from phosphatidic acid. Arachidonic acid can be metabolized by cyclooxygenase to form prostaglandins, by lipoxygenases to form leukotrienes, hydroxyeicosatetraenoic acids (HETEs) and hepoxylins, or by epoxygenases to form epoxyeicosatrienoic acids. Many of these metabolites are neuro- and vasoactive. Arachidonic acid and some metabolites can increase $[Ca^{2+}]_i$ after diffusion to surface receptors on adjacent cells and others can modulate the formation of cyclic GMP or cyclic AMP. Lysophosphatidyl choline may also act as a second messenger, for example, by stimulating cyclic AMP phosphodiesterase, and, when acetylated, becomes platelet-activating factor.

Cyclases

The cyclic AMP cascade is the best-studied signal transduction system (for review see Kebabian, 1992). Cyclic AMP is formed by the enzyme adenylyl cyclase, which is linked to many different cell surface receptors via G proteins ($G\alpha_s$ as positive regulator, $G\alpha_i$ as a negative regulator). Cyclic AMP can then activate cyclic AMP-dependent protein kinase A (PKA), which phosphorylates specific proteins at serine and threonine residues. Cyclic AMP is broken down by cyclic nucleotide phosphodiesterases (CN-PDE).

There is considerable interaction between the phospholipid pathways and the cyclic AMP system. For example, the type I CN-PDE is activated by calcium and calmodulin, which would be available after IP_3-induced mobilization of intracellular calcium stores. Another PDE may be activated by lysophosphatidyl choline. Cyclic AMP signals may also be increased by arachidonic acid (Axelrod, 1990), and protein kinase A can decrease the activity of some forms of phospholipase C.

Cyclic GMP is also a second messenger, but its regulation is quite different from that of cyclic AMP. Cyclic GMP can be produced by activation of receptors with intrinsic guanylyl cyclase (GC) activity, for example, the receptors for atrial natriuretic peptides (for review see Chinkers and Garbers, 1991). These receptors have a single transmembrane region and intrinsic GC activity, often termed particulate GC.

Cyclic GMP can also be formed by a soluble GC (sGC), which is a heme-containing heterodimeric enzyme not associated with the membrane. Its main activators are nitric oxide (NO) and carbon monoxide (CO), which interact with the heme moiety. Nitric oxide can be formed in many cells expressing either a constitutive nitric oxide synthase (NOS) or an inducible NOS, and freely diffuses to adjacent cells where it can activate sGC (for review see Nathan, 1992). Carbon monoxide is produced by the activation of heme oxygenases (for review see Maines, 1993). Cyclic GMP activates protein kinase G (PKG), which, among other actions, phosphorylates a Ca^{2+} pump (Yoshida et al., 1991), permitting a decrease in $[Ca^{2+}]_i$, which mediates the relaxation of vascular smooth muscle in response to endothelium-derived NO (see Figure 34-3).

GENERATION OF SECOND MESSENGERS IN GLIAL CELLS

Phospholipase C

As demonstrated by immunohistochemistry, astrocytes in culture contain high levels of PLCδ and lower levels of PLCβ and γ, relative to oligodendrocytes or neurons (Mizuguchi et al., 1991), and this is consistent with the finding of PLCδ-positive astrocytes *in situ* (Choi et al., 1989). Developmentally, a small population of astrocytes in various regions of newborn rats, including cerebellar radial glia, show weak immunopositivity for PLCβ and -γ, while these forms are limited to white matter and hippocampus in adults. Cerebral astrocytes show PLCδ immunoreactivity beginning in the second postnatal week, which is distributed throughout the brain in the adult (Yamada et al., 1991). Since PLCγ is linked with growth factor receptors, these findings suggest that PLCγ may be involved in the proliferation of radial glia, which are thought to guide neurons in developing cerebellum. However, these cells switch to the PLCδ isoform in the adult, consistent with a role involving astrocyte responses to neurotransmitters via G protein-linked receptors.

A vast number of cell surface receptors have been linked to polyphosphoinositide hydrolysis in astrocytes. These include muscarinic cholinergic and α_1 adrenergic receptors (Pearce et al., 1985), as well as endothelin receptors (Chuang et al., 1991), bradykinin and vasopressin receptors (Cholewinski and

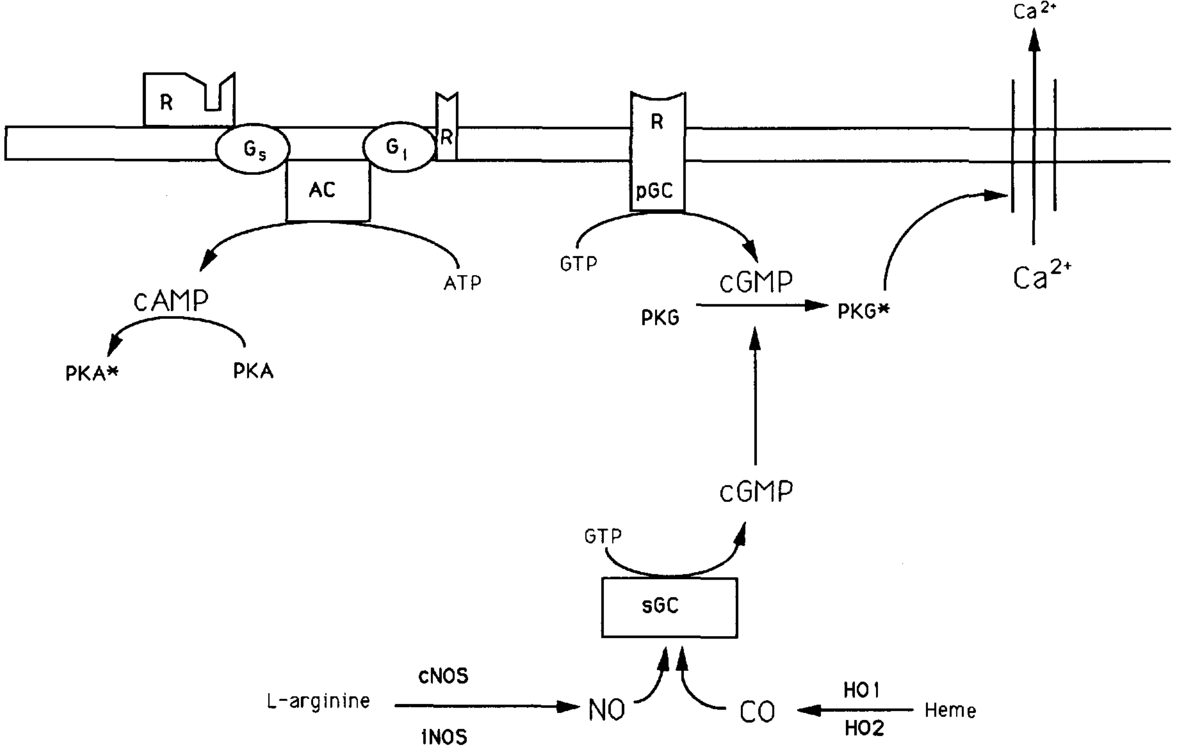

FIG. 34-3. Roles of cyclases in intracellular signaling. Adenylyl cyclase (*AC*) is under positive (G_s) or negative (G_i) control by G protein coupled receptors. The resulting cyclic AMP (*cAMP*) activates cAMP-dependent protein kinase (*PKA*). Particulate guanylyl cyclase (*pGC*) is receptor-associated, while soluble guanylyl cyclase (*sGC*) can be activated by nitric oxide (*NO*) produced by constitutive or inducible nitric oxide synthase (*cNOS, iNOS*) or by carbon monoxide (*CO*) produced by heme oxygenases (*HO*). Cyclic GMP (*cGMP*) produced by GCs activates cGMP-dependent protein kinase (*PKG*), which can influence calcium levels within the cell, possibly by phosphorylating a calcium transporter. See text for details.

Wilkin, 1988), prostaglandin $F_{2\alpha}$ receptors (Kitanaka et al., 1991), NK1 (substance P) receptors (Beaujouan et al., 1990), metabotropic glutamate receptors (Pearce et al., 1990), and P_{2y} purinergic receptors (Pearce et al., 1989). Calcium ionophores and histamine also evoke inositol phospholipid turnover in astrocyte cultures. Their effects are acutely sensitive to the presence of external calcium and may be due more to the hydrolysis of PI and PIP than PIP_2 (Pearce and Murphy, 1988).

As in other cells, there is crosstalk between second-messenger systems in glia. The polyphosphoinositide turnover induced by glutamate in astrocyte cultures can be inhibited by increases in cyclic AMP, which appears to be acting at or distal to a G protein, but not at the PLC itself (Robertson et al., 1990).

Phospholipase D and Phospholipase A_2

There is now evidence for PLD activity in astrocytes, although the endogenous regulation and consequences of its activation are not clear. Phorbol esters, which activate protein kinase C by mimicking diacylglycerol, activate PLD in cultured astrocytes (Bruner and Murphy, 1990b; Gustavsson and Hansson, 1990), as do calcium ionophore and thapsigargin that increase $[Ca^{2+}]_i$. Receptors linked to PLD in astrocytes have yet to be demonstrated conclusively. The calcium-induced PLD activity is inhibited by downregulation of PKC via long-term phorbol ester treatment, suggesting that the rise in $[Ca^{2+}]_i$ produced by these agents activates a PKC, which then activates PLD (Bruner and Murphy, 1990b).

There is also evidence for astrocyte PLA_2 activity, isoforms of which are under very diverse control. Activation of a P_{2y} purinergic receptor in primary astrocyte cultures leads to production of arachidonic acid and thromboxane (Bruner and Murphy, 1990a; Bruner and Murphy, 1993; Pearce et al., 1989). Initially, this was presumed to be via a pathway of calcium-activated PLA_2. The rise in $[Ca^{2+}]_i$ could be due either to PLC via IP_3-induced calcium mobilization from internal stores or through calcium influx induced by ATP (Neary et al., 1988b). However, Bruner and Murphy (1993) found that pertussis toxin, which at low concentrations completely inhibits ATP-stimulated thromboxane formation, only affects IP turnover at much higher concentrations, suggesting that the prostanoid production is not dependent on PLC activation but instead results from a direct linkage of the P_{2y} receptor with PLA_2. These results explain why only ATP and ADP evoke thromboxane release from astrocytes, although many other agonists also activate PLC. Additional evidence for the separation of ATP-induced PI turnover and AA production comes from the observation that serotonin inhibits ATP-stimulated AA production but actually increases ATP-evoked IP turnover (Murphy and Welk, 1990).

Despite a direct receptor linkage of PLA_2, the enzyme is still dependent on the presence of calcium. The interaction with a G protein simply seems to lessen the degree of calcium dependence when compared with ionophore-induced activation (Bruner and Murphy, 1993). Since phorbol esters also induce calcium-dependent prostaglandin production from astrocytes (Jeremy et al., 1987), PLA_2 appears also to be regulated by protein kinase C.

Expression of a secreted form of PLA_2 can be stimulated by inflammatory factors such as endotoxin (lipopolysaccharide), interleukin (IL-1), and tumor necrosis factor (TNF-α) in astrocyte cultures (Oka and Arita, 1991). These agents use different pathways of activation, since lipopolysaccharide induction is sensitive to dexamethasone and inhibition of protein kinase C, while the TNF-induced production is enhanced by increases in cyclic AMP. Additionally, in C_6 glioma cells that have astrocytic properties, there is evidence for a $GABA_A$ receptor linked to PLA_2. Activation of this PLA_2 can be enhanced by benzodiazepines but is not associated with a picrotoxin-sensitive chloride channel (Majewska and Chuang, 1985).

Adenylyl Cyclase

Adenylyl cyclase (AC) is under both positive and negative regulation in astrocytes. For example, isoprenaline or norepinephrine stimulates cyclic AMP production via interaction with β-adrenergic receptors, the classical AC linked receptor (Marin et al., 1990; Ruck et al., 1991). However, if these two agonists are used together, the α_2 activation by the nonspecific actions of norepinephrine actually attenuates the AC activation by the specific β-agonist isoprenaline (Ruck et al., 1991). Additionally, in cultures of mixed type I and type II rat astrocytes, high levels of isoprenaline resulted in a decrease in cyclic AMP production due to activation of α_1 receptors, but this effect was not seen in pure type I cultures (Ruck et al., 1991). By contrast, in mouse striatal astrocyte cultures, when α_2 and dopamine responses were blocked, α_1 activation by norepinephrine potentiated the β-induced cyclic AMP response, as did activation of α receptors with methoxamine. Such potentiation of the β response was dependent on external calcium (Marin et al., 1990). These obser-

vations again demonstrate the crosstalk between different second-messenger systems and illustrate how the second-messenger response induced by a particular agonist can be modulated by different signals received by the astrocyte concurrently.

Adenosine receptors also control AC in astrocytes. Cyclic AMP levels in primary astrocyte cultures are increased with adenosine analogues with a rank order of potency, suggesting activation of an A_2 receptor. Adenosine analogues can inhibit cyclic AMP production by isoproterenol with a rank order of potency, suggesting activation of an A_1 receptor (Murphy et al., 1991). Histamine also appears to activate AC via H_2 receptors in type I but not type II astrocytes in culture (Kubo et al., 1991).

Finally, astrocyte AC can be influenced by prostaglandins and opioids. Prostaglandin E_1 causes an increase in cyclic AMP in astrocyte cultures from embryonic rat striatum, cortex or brainstem. In each case cyclic AMP accumulation could be slightly attenuated by a δ opioid receptor agonist but not a μ receptor agonist, with the least effect in brainstem cultures (Eriksson et al., 1991). The AC/cyclic AMP appears to be under a multitude of controls, hence the response of an astrocyte to a particular AC stimulatory agonist will depend not only on what type of astrocyte it is, the anatomic location, and developmental stage, but also what other signals are being received by the cell concurrently.

Guanylyl Cyclases

As demonstrated by cytochemistry, astrocytes contain both guanylyl cyclase (GC) and cyclic nucleotide phosphodiesterase (Poeggel et al., 1991). The GC activity results from the presence of both particulate and soluble forms of the enzyme. Receptors for atrial natriuretic peptide, which contain intrinsic GC activity, cause cyclic GMP increases in cultures of astrocytes from neonatal rat hypothalamus and brainstem. The effectiveness of various peptides indicates that these receptors are primarily of the "A" type, which are most responsive to atrial and brain natriuretic peptides and less responsive to atriopeptin II (Sumners and Tang, 1992).

Astrocyte cultures respond to the nitric oxide donor sodium nitroprusside and to NO production from cerebellar granule cells with an increase in cyclic GMP (Kiedrowski et al., 1992). Both effects are presumably due to an activation of the soluble GC. Besides activation by exogenous NO, the soluble GC in astrocytes can also be activated by NO produced by the cells themselves (for review see Murphy et al., 1993). Agullo and Garcia (1992) have demonstrated that α_1 adrenergic, dopaminergic, bradykinin, vasoactive intestinal peptide, adenosine, and metabotropic glutamatergic receptors mediate cyclic GMP production in astrocytes, which is sensitive to inhibition by *N*-substituted analogues of L-arginine, indicating the production of NO by a calcium-dependent constitutive nitric oxide synthase. Additionally, long-term treatment with lipopolysaccharide and cytokines can induce a distinct nitric oxide synthase in astrocyte cultures and C_6 glioma cells (Galea et al., 1992), and in microglia (Banati et al., 1993). This calcium-independent nitric oxide synthase is also capable of increasing cyclic GMP levels in the cells in an arginine-dependent manner (Simmons and Murphy, 1992, 1993) via action on the soluble GC. Therefore, cyclic GMP levels in astrocytes can be modulated on a short-term basis via activation of atrial natriuretic peptide receptors, or soluble GC via NO from adjacent cells and astrocytes themselves. On a more long-term basis, induction of nitric oxide synthase by inflammatory mediators subsequently activates soluble GC.

TARGETS FOR SECOND MESSENGERS IN GLIAL CELLS

Evidence for a Nonneuronal Inositol Trisphosphate Receptor

Considerable evidence suggests that activation of receptors on astrocytes linked to PLC generates IP_3 and promotes mobilization of intracellular calcium (Finkbeiner, 1993; see Chapter 17, this volume). Thus, an IP_3 receptor must be present in these cells, and an autoradiographic study (Hösli and Hösli, 1991) has revealed binding of (^{3}H)IP_3 to astrocytes as well as to neurons. However, distribution of this homotetrameric receptor (subunit molecular weight of 260 kD) was described initially by autoradiography as being neuronal (Worley et al., 1989). The IP_3 receptor is one of the largest proteins to have been sequenced and is suggested to comprise eight transmembrane domains at the C terminus (for reviews see Berridge, 1993; Ferris and Snyder, 1992). However, through the use of the polymerase chain reaction, it is now recognized that there are distinct transcripts for the IP_3 receptor, probably derived by alternative splicing (Danoff et al., 1991; Meldolesi, 1992). A shorter nonneuronal form lacks a 120-nucleotide insert between the two protein kinase A phosphorylation consensus sequences. This form predominates in fetal brain and peripheral tissues, and is present in C6 glioma cells.

The IP_3 receptors share considerable homology in some of the transmembrane domains with the much

larger ryanodine receptors that mediate calcium-induced calcium release in neurons and in cardiac/skeletal muscle (Taylor and Marshall, 1992). While dantrolene, which blocks calcium-induced calcium release, inhibits calcium waves in glial cells (Charles et al., 1993), cytochemical evidence for ryanodine receptors is still wanting.

Protein Kinases Are Expressed in Glial Cells

Clearly, activation of phospholipase C in astrocytes liberates diacylglycerol. The observation that phorbol esters influence a range of astrocyte properties which include enzyme activity (Jeremy et al., 1987; Pearce and Murphy, 1988; Bruner and Murphy, 1990b; Gustavsson and Hansson, 1990), gap junction formation (Enkvist and McCarthy, 1992), expression of early response genes (Chapter 35, this volume), and volume regulation (Bender et al., 1992) implies the presence of protein kinase C (PKC), and the enzyme has been demonstrated both biochemically (Neary et al., 1988a) and by autoradiography (Hosli and Hosli, 1991). Ten subspecies of PKC have been described (Azzi et al., 1992; Hug and Sarre, 1993). Types I (γ), II (β), and III (α) are found in brain (Todo et al., 1990; Tanaka and Saito, 1992), and type III is almost exclusively localized in astrocytes and oligodendrocytes (Hidaka et al., 1988; Komoly et al., 1991) as revealed by immunocytochemistry.

The type II regulatory subunit of PKA is prominent in astrocytes (Loffler et al., 1985). Evidence for PKA function in these cells includes the effects of cyclic AMP on phosphorylation of glial fibrillary acidic protein (GFAP) and vimentin (McCarthy et al., 1985; Mobley and Combs, 1992), in the regulation of glycogen turnover (Cambray-Deakin et al., 1988) and membrane receptors (Robertson et al., 1990), and recently in the suppression of nitric oxide synthase induction (Feinstein et al., 1993).

Calmodulin-dependent kinase activity and calmodulin kinase II-like immunoreactivity have been described in astrocytes (Bronstein et al., 1988; Scholtz et al., 1988; Babcock-Atkinson et al., 1989). In neurons, calmodulin kinase phosphorylates proteins involved in neurotransmitter synthesis and release. In astrocytes, potential protein substrates include cyclic nucleotide phosphodiesterase, GFAP and vimentin, glycogen synthase, the IP_3 receptor, and PLA_2 (Schulman and Hanson, 1993). Recent evidence suggests involvement of this enzyme in the regulation of astrocytic volume that follows cell swelling (Bender et al., 1992).

Although cyclic GMP is known to have a variety of effects in cells of the nervous system, including modulation of ion channels and transmitter release (Garthwaite, 1991), the role of PKG in astrocytes has been little studied as compared with cardiac muscle or platelets (Butt et al., 1993). Clearly, with the observations that cyclic GMP levels in astrocytes are regulated both by peptides (Sumners and Tang, 1992) and by NO (Agullo and Garcia, 1992; Simmons and Murphy, 1992, 1993), this is an area that demands attention.

Second Messengers Can Reach Distant Targets

The actions of some second messengers may extend beyond the cell in which they are generated. This results from the fact that astrocytes may be coupled via gap junctions to each other or to neighboring cells, such as oligodendrocytes (see Chapter 19), and the best example is that of calcium moving through a syncytium of astrocytes (see Chapter 17). In addition, the chemical nature of a number of second messengers endows them with the ability to leave the cell of origin and enter neighboring cells. One obvious candidate is arachidonic acid, which is liberated by the activation of phospholipase A_2 and leaves astrocytes (and indeed all cells) in large amounts unmetabolized. Not only can this arachidonic acid serve as a substrate for metabolism through the various eicosanoid synthesis pathways in near-neighbor cells (neurons and cells of the vasculature), but it has been shown to directly modulate phospholipase C activity (Murphy and Welk, 1989).

Other diffusible messengers of particular note are NO and CO. The evidence for production of NO either by acute activation of a constitutive nitric oxide synthase or transcriptional induction of nitric oxide synthase in astrocytes has already been presented. The diffusibility of NO makes it likely that this messenger signals events to neighboring cells. Acute release of NO from perisynaptic astrocytes could result in modulation of synaptic transmission. Tonic release from astrocytes in which nitric oxide synthase has been induced could have profound and long-lasting effects on adjacent vasculature, modulating tone and permeability. Recently, the presence of a heat-shock-induced heme oxygenase (HO 1) in astrocytes has been reported (Ewing and Maines, 1991). Whether a constitutive form of the enzyme (HO 2) found in neurons is also present in glial cells remains an open question. Production of CO by HO, and of NO by NOS, could then activate soluble guanylyl cyclase locally in the cell of origin or in adjacent cells. In addition, via interactions with the array of heme-containing proteins found in cells, CO and NO can potentially modulate a variety of functions.

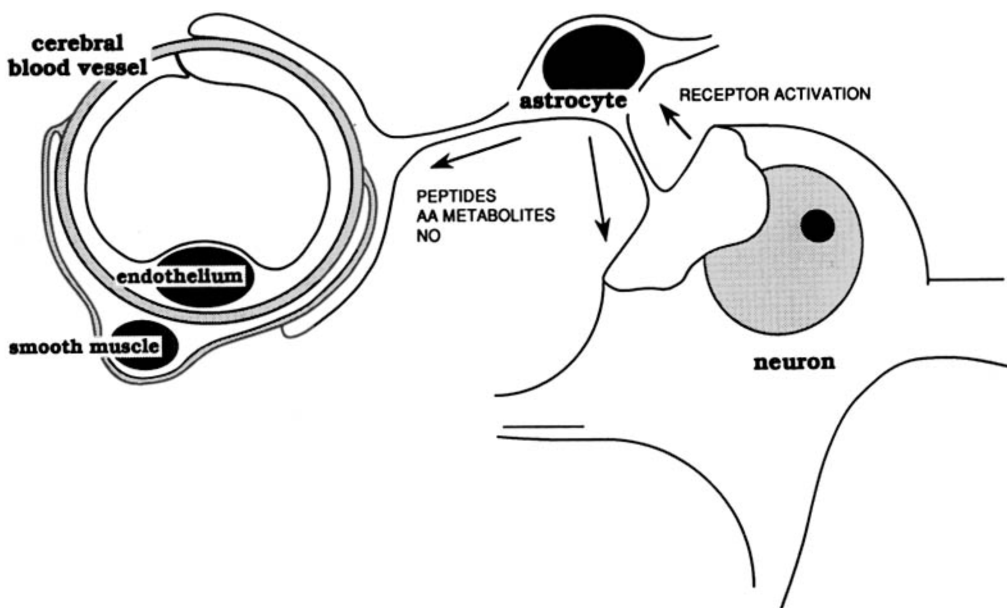

FIG. 34-4. Potential interactions between astrocytes, neurons, and the cerebral microvasculature. Signal molecules released from neurons activate specific receptors on adjacent astrocytes. Depending on the brain region and the receptor-activated second-messenger system, astrocytes release peptides (growth factors, endothelins, enkephalins, somatostatin), arachidonate (*AA*) metabolites (prostanoids, leukotrienes) or nitric oxide (*NO*). These signal molecules, in turn, can modulate functions of cells of the vessel wall (flow, permeability). In addition, the release of such agents from astrocytes can modulate neuronal metabolism and synaptic transmission.

SUMMARY

In the last few years the evidence for receptor-activated changes in glial cell metabolism has become incontrovertible. From the initial descriptions of binding sites for neurotransmitters on glial cells in culture we can now trace the signaling mechanisms by which particular agonists modulate specific glial cell functions. Through *in situ* autoradiography and now hybridization techniques we can also be assured that the expression of a particular receptor-effector pathway is not an artifact of the culture dish. The view of glial cells that emerges is that they have the capacity to function in a complex interaction with neurons and neighboring vessels (see Figure 34-4). The extent to which these proposed interactions occur *in vivo,* the degree to which classes of glial cells (e.g., astrocytes) are functionally heterogeneous, and the environmental signals that create and maintain this diversity—all this has yet to be revealed.

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35 Early-response gene expression in glial cells

ALARIC ARENANDER AND JEAN DE VELLIS

For many years a gap existed in our understanding of the molecular mechanisms that mediated the transfer of short-duration extracellular signals into long-lasting phenotypic responses of glial cells. Most of these cellular responses were known in terms of events that appeared several hours after a cell was activated by a specific ligand. For example, one of the best studied genomic responses was the induction of the glycerol phosphate dehydrogenase (GPDH) gene and the subsequent increase in cytoplasmic enzyme activity (Kumar and de Vellis, 1988). GPDH induction occurs after treatment of oligodendrocytes or C6 glioma cells with glucocorticoids and/or activators of protein kinase A (PKA). Although both of these signaling pathways are activated within a short time, on the order of minutes, the increase in GPDH message and protein is seen only after several hours. A hint of the processes that occurred in this 1- to 2-hour gap came from experiments showing that ligand-mediated GPDH transcription was prevented by cycloheximide, an inhibitor of protein synthesis. Thus, the activation of GPDH required an early event coupled to the signaling pathway cascade that involved the synthesis of new protein. A number of other genes expressed in glial cells, such as transferrin, transin, myelin basic protein (MBP), and proteolipid protein (PLP), also share this property.

At about the same time, a set of genes was discovered that displayed very rapid expression kinetics. These are called early-response genes (ERGs) to distinguish them from the late-response genes (LRGs) such as GPDH. These genes are also called immediate early genes in analogy to viral expression patterns or primary response genes, since they precede the induction of secondary response genes (for review see Arenander and de Vellis, 1993; Arenander and Herschman, 1993). At the end of this first decade of study, these genes are regarded as key intermediaries in nearly every signaling cascade process and represent the molecular mechanism that bridges the gap. Their rapid and coordinated expression is required for the expression of many genes that code for phenotypic proteins and thus are considered necessary components in the expression of GPDH and a number of other glial-specific proteins in the nervous system. Their role in cellular responses is so widespread and complex that they are often referred to as the "third-messenger" system of signal transduction. In this chapter, we will review the research on ERG expression in glial cells during development and in the adult nervous system. The current focus of ERG research is how to integrate all the numerous ERG proteins into a coherent picture of the complex signaling process that leads to cell type-specific phenotypic response.

CLASSES OF EARLY-RESPONSE GENES

When a cell is exposed to an environmental agent, specific receptor-coupled signaling pathways are activated, which very quickly lead to altered processes in the cell nucleus. As a result, these pathways mediate the rapid onset of gene transcription. ERG mRNAs are then translated into proteins that are targeted to specific cellular locations and functions (see Figure 35-1). Four main types of ERG products are illustrated. Most of the ERG proteins discovered to date are transported to the nucleus and function as transcription factors. This chapter deals primarily with this class of genes that are part of the early response of the cell. As seen in Figure 35-1, the ERG proteins functioning as transcription factors coordinate the next or "late" round of genomic expression that contributes to a long-lasting phenotypic response. Of considerable interest are the types of ERG proteins that serve metabolic or structural roles in the cell or are integrated into the cell plasma membrane and serve as receptors for extracellular ligands that play important roles in glial development. A final type of ERG protein is secreted as a signal molecule to influence the behavior of neighboring cells. The different classes of protein encoded by ERGs are also listed in Table 35-1, based on message kinetics along with some examples.

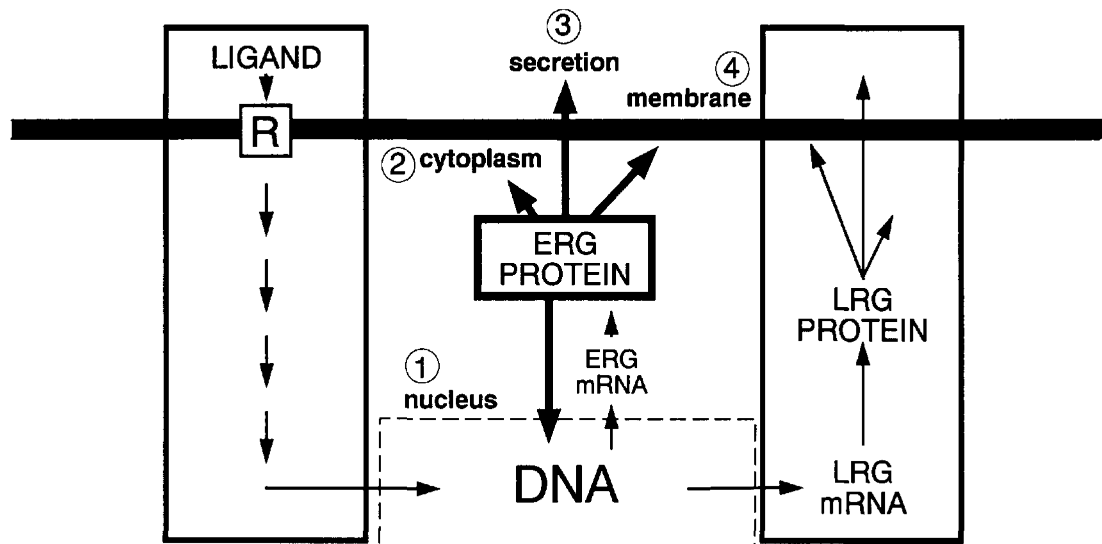


FIG. 35-1. Early-response genes (ERGs). These genes represent a new type of genomic program, acting as crucial intermediaries or "third-messenger" molecules, linking short-lasting extracellular signals to long-term gene expression and phenotypic change. The rapid expression of these genes lead to the appearance of proteins in many compartments of the cell. Shown here are the four main types of ERG products. Most of the ERGs discovered to date encode for proteins that return to the nucleus and function as transcription factors, regulating the kinetics of their own transcription and of the late-response genes (*LRGs*).

It is evident that the ERGs encoding for transcription factors demonstrate the most rapid and transient kinetics of both the message and protein and are termed class I ERGs. Two subtypes of ERGs can be delineated in this class of nuclear regulatory proteins: Class IA mRNA ERGs have very rapid expression kinetics with a very short half-life, while the expression of class IB mRNA is delayed somewhat, with a slightly longer half-life. The characteristic rapid induction kinetics and equally rapid kinetics of degradation of class I ERGs, yield very narrow windows of message accumulation and thus relatively short periods of protein accumulation, resulting in a temporally constrained period of transcriptional control of LRGs following an extracellular signal. These short, well-defined periods allow the cell to express a very powerful, but temporally limited, mode of communication to provide precise and specific control over its genetic programs. These ERG kinetics, therefore enable the cell to quickly "reset" its information gathering and processing programs and allows its genetic programs to remain sensitive to rapidly changing events in the environment. It also provides an extremely complex and efficient means of integrating the demands of the environment with the needs of numerous cellular

TABLE 35-1. *Early-Response Gene Classification*

	mRNA				
Class	Onset	Peak	Offset	Half-Life	Example
IA[a]	5–30 min	30–60 min	2 h	10–15 min	c-*fos*, c-*jun*, *egr1*
IB[a]	30–60 min	2 h	4–8 h	30–60 min	c-*myc*, *fra1*
II[b]	30–60 min	2 h	4–8 h	30–60 min	*IL-6, JE, KC,* fibronectin
III[c]	1–2 h	4–6 h	4–8 h	>2 h	$p75^{NGFR}$, $p140^{trkA,B,C}$, *glucose transporter*
IV[d]	30 min–5 h	1/2–2 h	12–24 h	>3 h	*Actin, vimentin, NOS, PGS*

NOS, nitric oxide synthetase; PGS, prostaglandin synthetase.
[a]Nuclear regulatory proteins.
[b]Secreted proteins.
[c]Membrane proteins.
[d]Cytoplasmic proteins.

processes. Additional characteristics of ERG transcription factors are discussed below.

In contrast, the remaining classes of ERG proteins, encoding for cellular or membrane proteins, display generally slower kinetics, most likely appropriate for each of their functional roles in the cell specific response. The expression of the cytokine IL-6 has been extensively studied in glial and in immune cell types. The rapid kinetics of message induction, independent of protein synthesis, mark IL-6 as a class II ERG encoding secreted protein. Class III ERGs encoding membrane receptors include the expanding family of neurotrophin receptor proteins. Considerable work has been conducted in primary and clonal glial cells (Kumar et al., 1990, 1993) showing the ligand-dependent induction characteristics of the low-affinity (p75 NGFR) and the associated *trk* family of receptors (p140 *trk*A, B, C, see also Chapter 29). To date, only *trk*B and *trk*C have been found expressed in glial cells. Of the class IV ERGs, a ligand-inducible form of Prostaglandin synthetase II (PGS) (originally named TIS10) has been cloned and its expression studied in glial and other cell types (Arenander et al., 1989). Nitric oxide synthetase (NOS) also has an inducible form, and its kinetics and regulation are actively being examined (Bruner et al., 1993). Vimentin and actin are found in glia.

ENCODING TRANSCRIPTION FACTORS IN GLIAL CELLS

Many ERGs encode for transcription factors. Transcription factors, in general, can be classified based on the motifs of their protein domains that permit sequence-specific DNA binding and/or dimerization. Table 35-2 lists the main classes of transcription factors and some examples of each class. The number of classes, that is, unique motifs, is expected to increase over the next few years of research, as well as the membership of each family. At present, about 50 proteins have been examined for their genomic sequence, dimerization characteristics, DNA binding specificity, and transactivating potential (for review see He and Rosenfeld, 1991). Members of each of these classes, with a few exceptions, have been described in the nervous system, particularly in glial cells.

TABLE 35-2. *Early-Response Genes Encoding Transcription Factors*

Class (Domain)	Gene
Leucine zipper	*fos*, *fos*B, *fra*1, 2 *jun*, *jun*B, *jun*D *C/EBP*α,β,δ
Zinc finger	*egr* 1, 2, 3, 4 *NGF1C* *N10*
Helix-loop-helix	c-, N-, L-*myc* *HES1* *Id3*
rel	*rel*B
POU	*scip*

Three sets of research findings have influenced our efforts over the last few years (Arenander and de Vellis, 1993). *First,* it became apparent that many ERGs are induced when cultured glia are exposed to a ligand (Arenander and de Vellis, 1993). These results were consistent with the evidence rapidly accumulating for nonneural cell types that ERGs function in a combinatorial fashion to carry out their task as a third-messenger system. Although some cases of cell type-restricted expression have been reported (Herschman, 1991), these findings are an exception, rather than the rule, and the encoding of environmental information was not detected in differential patterns of restricted expression. Thus, nearly every member of every family is induced following ligand stimulation *in vitro*. Specificity of the encoded information therefore lay in the realm of differential ERG expression kinetics of message and protein and of posttranslational protein modification.

Second, the data from our laboratory and from a few published reports suggested that the expression pattern *in vivo* was highly restricted, in marked contrast to the cultured cells used in most research. This suggests ERG expression can be developmentally restricted and thus unique patterns of ERG expression may exert important influences on lineage decisions during glial development.

Third, an important issue that encourages multiple probing is the well-known variable dimerization characteristic of transcription factors. Research has demonstrated that a major mode of regulation of ERG transcription factor availability and promoter transactivation potential lies in heterodimer formation. Alternative binding partners can decide whether a factor is active and the degree of its transcriptional activation. The phenomenon becomes even more complex when one considers dimer formation not only within families but between families. Best known are the interactions among the *fos* and *jun* families. More recently, the HLH proteins have been shown to exhibit considerable heterodimerization with dramatic results (see below). For these reasons, we have expanded our ERG investigation to include members of five families of ERGs shown in Table 35-2.

LIGAND-MEDIATED TRANSCRIPTIONAL ACTIVATION OF EARLY-RESPONSE GENES

Research has examined how ERG proteins bind to specific regions of many late-response gene promoters, inducing the transcription of these genes. The mechanism responsible for the transcriptional activation of the ERGs themselves, however, until recently has remained unclear. *First,* many ERG genomic clones have been sequenced, indicating the nature of their promoter elements and configuration, indirectly suggesting specific modes of control. *Second,* some research has begun to examine the signaling cascade directly controlling the specific regulatory factors responsible for ERG promoter activation. The reason that ERGs, especially class I ERGs, display such rapid-onset kinetics is because preexisting, inactive ERG proteins located in the nucleus are quickly activated and turn on ERG transcription without much delay. Thus, ERG proteins are used to "kick-start" their own transcription activation.

The best understood example of control via post-translational modification of preexisting, inactive ERG proteins located in the nucleus is depicted in Figure 35-2. Under quiescent, basal conditions, the low levels of nuclear c-Jun possess a phosphorylation pattern that inhibits DNA-binding ability and hence prevents transcriptional activation. Exposure of the cell to ligand activates various intracellular signaling pathways leading to altered activity of various nuclear protein kinases and phosphatases. Activation of protein kinase C (PKC) by the phorbol ester (TPA) leads to activation of phosphatases that rapidly dephosphorylate two inhibitory C-terminal sites near the DNA-binding domain of c-Jun. This change in phosphorylated state permits efficient binding of c-Jun dimers to the TPA-responsive element within the promoter of c-Jun and other ERGs. c-Jun binds as a homodimer, since the initial lack of significant amount of c-*fos* disallows heterodimer formation.

Thus, the equilibrium between various nuclear kinases and phosphatases appears to be a major locus of control over the transactivating ability of c-Jun and, most likely, other ERG-encoded transcription factors. Thus, the ligand-induced shift in nuclear dominance from the constitutively expressed casein kinase II (CKII) to phosphatase and other kinases, alters the phosphorylated state of c-Jun and leads to strong induction of c-Jun responsive genes such as c-Jun itself, c-*fos,* and other ERGs.

The transient nature of transcriptional activation, characteristic of ERG expression, is due, in part to feedback control. For example, Fos is known to negatively regulate its own expression. Since c-Jun is a positive autoregulator (positive feedback), inhibitory control over c-Jun expression may depend upon an-

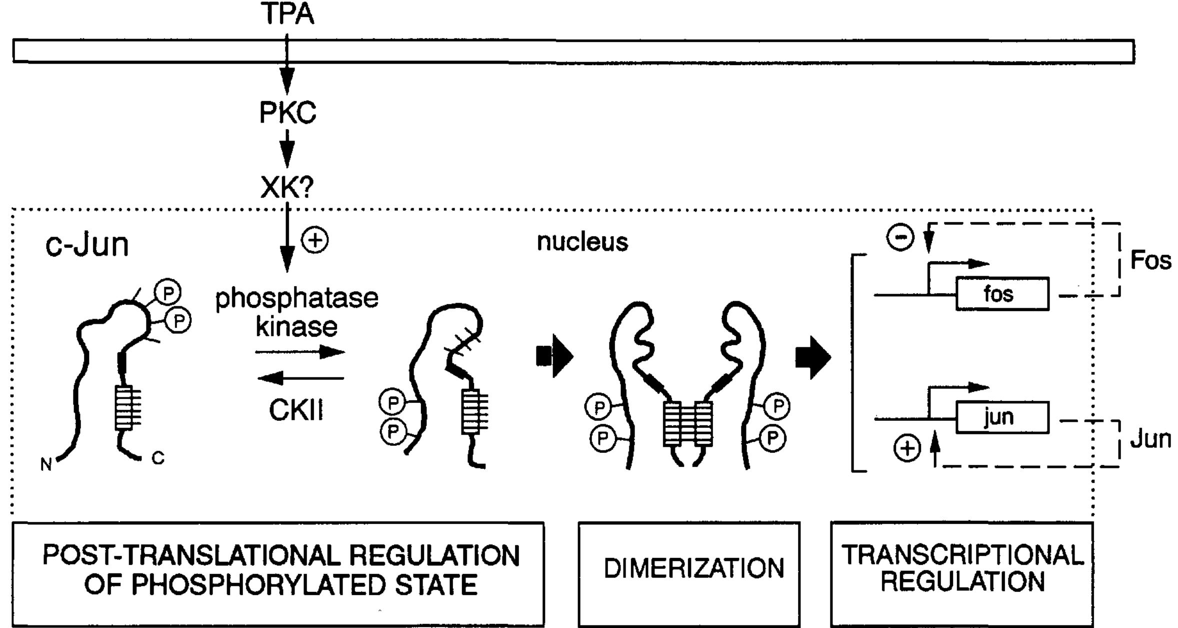


FIG. 35-2. Ligand-induced modification of ERG proteins and ERG transcription. The very rapid transcriptional induction of ERGs is due to the rapid modification of preexisting ERG proteins, which remain in an inactive state in the nucleus until the activation of the appropriate signaling pathway leads to an altered equilibrium between kinase and phosphatase activity. See text for details. The DNA-binding domain of c-Jun is shown as a stippled box. *CKII,* casein kinase II; *PKC,* protein kinase C.

other inhibitory protein which binds to the amino-terminal portion of c-Jun to reduce its ability to be a potent transcriptional activator. c-Jun can also be modified by phosphorylation of its two N-terminal sites by various transforming oncogene proteins. This leads to augumentation of the transactivating potential of the c-Jun, but not its DNA-binding ability.

COMBINATORIAL CONTROL OF TRANSCRIPTION

Another remarkable feature of ERG expression is that not only is it rapid for the reasons discussed above, but that many different signaling agents are capable of inducing transcription. For example, the transcription of *c-fos* can be detected within 5 minutes of cell exposure to ligands, such as serum, polypeptide growth factors, TPA, and cAMP. All these agents and their corresponding signaling pathways appear to converge on at least one region of the *c-fos* promoter, the serum response element (SRE). This approximately 40-base pair (bp) region is essential for transcriptional expression of c-Fos. The SRE contains a 20-bp dyad symmetry element (DSE) and a 22-bp c-Fos AP-1 site (FAP). Data from nonneural cells is depicted in Figure 35-3, which shows that at least eight different subfamilies of transcription factors, many of which are ERGs, bind to overlapping regulatory elements in the SRE. The helix-loop-helix protein rE12 and the C/EBP-related factor rNFIL-6 bind to neighboring sites within the c-Fos serum response element. Thus, multiple signaling pathways appear to interact at the level of the ERG promoter to provide a highly complex, yet precise, mode of combinatorial control over ERG transcription. Since not all transcription factors can physically bind at one time to specific, yet, overlapping recognition sequences, it is evident that some selection or competition process must take place to determine which factors will access the SRE sequence. Such selection undoubtably influences the mode of control exerted by the SRE on c-Fos transcription.

As the level of protein and the degree of posttranslational modification of ERG proteins rapidly increase following the onset of ligand-induced transcription, crosstalk is expected, that is, many ERG proteins will influence each others promoter activity. Thus, it is important to note that ERG proteins, in addition to serving as transcription factors regulating late gene expression, are also key regulators of their own expression kinetics and functional state.

EXPRESSION PATTERNS IN ASTROCYTES

Single-cell analysis of cultured astrocytes by *in situ* hybridization suggests that ligand responsiveness of a population of astrocytes can display considerable heterogeneity (Arenander and de Vellis, unpublished data). However, differential induction of ERG

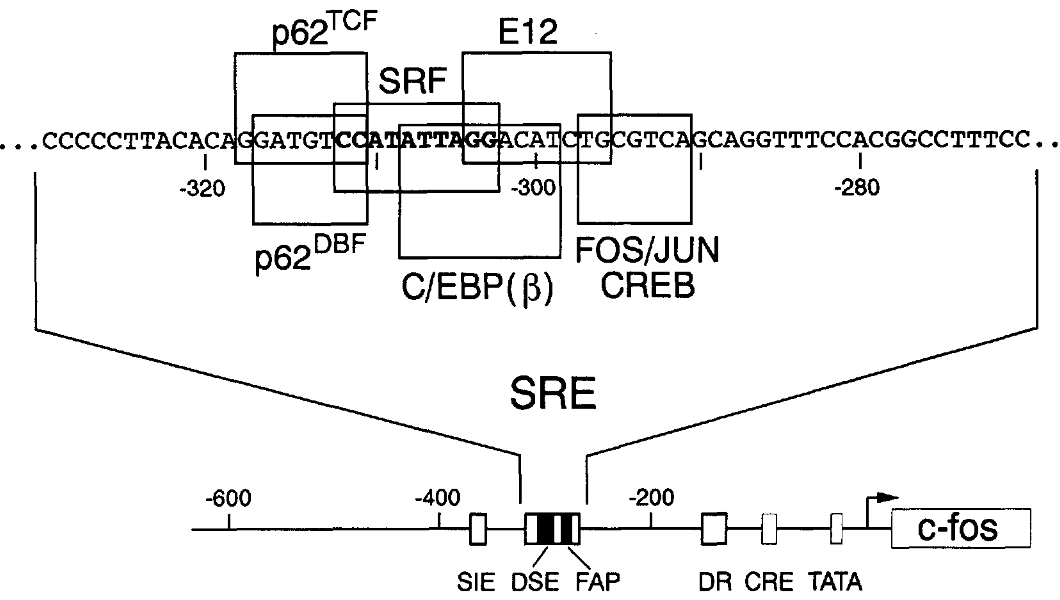


FIG. 35-3. ERG transcription factor interaction at the promoter: complexity of *c-fos* induction. The proximal part of the *c-fos* promoter is drawn below and the serum response element is enlarged above to the level of the nucleotide sequence. Note the sequence domains specific to each transcription factor overlap.

mRNA encoding transcription factors observed in cultured astrocytes appears due primarily to the variable degree of receptor expression in the cell population. In these types of experiments, TPA is used as a positive control, since all cells are expected to have PKC available for activation. Treatment of astrocytes with TPA induces both ERG mRNAs in nearly every cell. In contrast, stimulation by norepinephrine results in only about 60% of the cells expressing ERG mRNA. Since the TPA trials indicate the genes are inducible in all the cells, the response heterogeneity observed is most likely due to the known heterogeneity of nonadrenergic receptor expression in cultured astrocytes (Shao et al., 1993). Thus, ERG induction patterns represent an alternative, sensitive methodology for evaluating the cell type-specific expression pattern of functionally coupled receptors in astrocytes (Arenander and de Vellis, 1992).

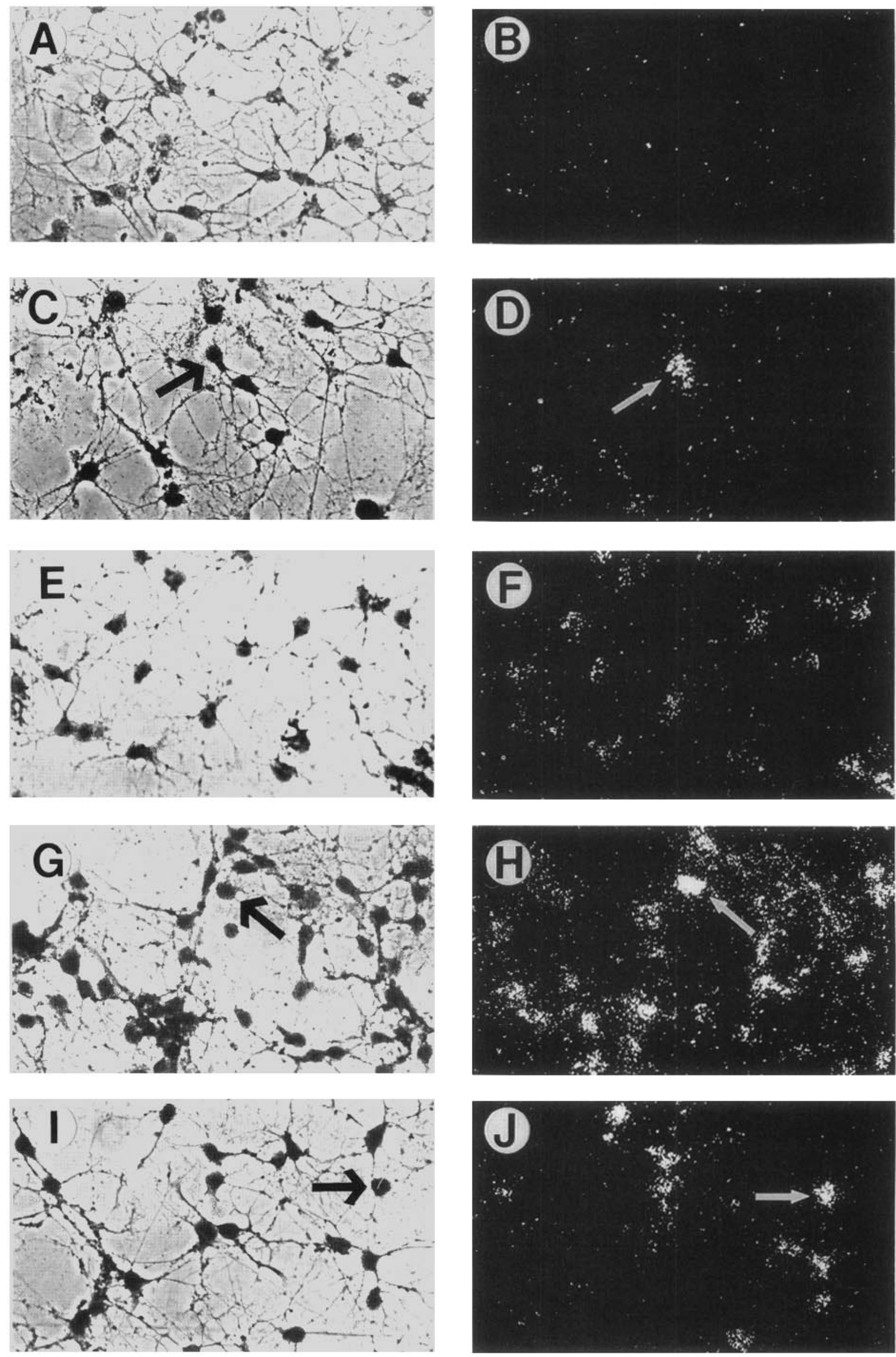
Note that the mode of receptor-signalling pathway coupling to gene induction differs, however, among the ERGs. Whereas ligand-mediated *NGFIB* induction is completely suppressed by a β-blocker, propranolol, the induction of *egr*1 is only slightly effected, suggesting a more complex mode of signaling-coupled regulation of transcription of this latter ERG. Although not shown here, the results of control compounds are active ligands capable of activating the signaling pathways and altering cell phenotype (Arenander and de Vellis, 1993). These antagonists, in the absence of agonist, are capable of not only binding to the receptor, but also perturbing receptor coupling to intracellular signal pathways, so as to induce a genomic response and increase the levels of ERG mRNA.

In contrast to class I ERGs, some of the other classes of ERGs do show restricted expression patterns *in vitro*. For example, mRNA for PGSII (TIS10; Class IV ERG) is detectable after an initial delay of several hours in astrocytes as seen in Northern data (Arenander et al., 1989). *In situ* data from similar experiments suggest that the induction occurs in only a small, but varying, percentage of cultured astrocytes (unpublished observations). The clustered induction pattern suggest the ability to express this inducible form of PGSII is restricted to rarely occurring (or low proliferation and/or survival) specific expanded "clones" of astrocytes in culture.

EXPRESSION PATTERNS IN OLIGODENDROCYTES

Rat oligodendrocytes can be cultured from postnatal cerebral cortices and grown under relatively controlled conditions with definable cell phenotypes based on cell morphology and specific immunocytochemical staining profiles. Since many ligands are known to alter the proliferation and/or progression through specific developmental lineage stages, the question arises whether ERG expression is a necessary component of the ligand-induced transduction process leading to phenotypic response and control of differentiation. As an initial, correlative approach, the inducibility of a variety of ERGs by some for the key extracellular signaling agents was studied. Figure 35-4 shows the degree of ERG mRNA accumulation in oligodendrocyte cultures following treatment with different ligands. In this case, to ensure detectability of message induction, the cells were treated with ligand plus cycloheximide, the latter added to permit accumulation of very high levels of message in any cell capable of responding to the ligand. Note that *NGFIB,* a member of the steroid receptor superfamily of inducible transcription factors, displays very low basal levels of expression, a characteristic of most class I ERGs. Treatment with TPA plus cycloheximide (TX) to activate the PKC system leads to a very high level of message expression in most of the cells. In contrast, bFGF plus cycloheximide (FX) activates a much smaller portion of the cell population and with varying degrees of message accumulation per cell. The restricted expression of ERG mRNA in FX-treated cells is therefore due primarily to heterogeneous peptide receptor expression. Treatment with cycloheximide alone does not result in much message accumulation.

In order to detect unique profiles of ERG expression associated with differential responsiveness to important environmental ligands, we routinely compare the induction pattern of a number of ERGs in response to several ligands. Thus the data shown in Figure 35-4 are part of a larger experiment that allowed a detailed comparison of ERG inducibility in these developing oligodendrocytes for several inducing agents (see Figure 35-5). Scoring of these cultures shows that most ERGs are "well-behaved," exhibiting standard characteristics of class I ERGs: no detectable basal expression in the presence or absence of cycloheximide. *NGFIB* is an exception, displaying an expression pattern that differs significantly from the other ERGs, including *egr*1, a member of the same zinc-finger transcription factor family (but different family from *NGFIB,* see Figure 35-3). TPA is clearly the strongest inducing agent for all the genes, both in terms of percentage of cells positive and in the strength of the signal (level of probe per cell). Note, however, about 15% of the cells exhibit no response to TPA, even though it is assumed that all cells possess PKC. These results differ from the nearly homogeneous induction response observed for TPA-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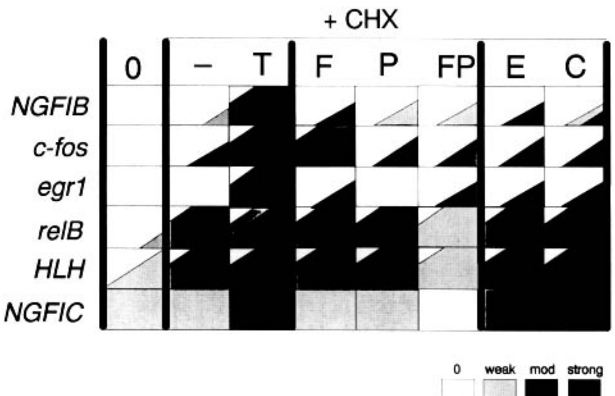

FIG. 35-5. ERG induction profile in developing oligodendrocytes. A number of agents known to influence oligodendrocyte development were tested for their effect on ERG mRNA induction as measured on the single cell level by *in situ* hybridization of oligodendrocyte progenitor cultures. This profile illustrates the unique pattern of ERG induction in these oligodendrocyte cultures. These data are derived from cell cultures shown in Figure 35-4 and from additional sister culture wells tested with other agents and examined for the expression of a number of different ERGs. Cell populations were scored for percentage positive cells for each condition and the degree of mRNA expression per cell based on a simple quantitative division into strong, moderate, weak or no level of mRNA expression. Conditions include two controls, no addition (O) or only cycloheximide (X). Cells were treated with inducing agent in the presence of X: *TPA(T)*, fibroblast growth factor (F), platelet-derived growth factor (*P*), epidermal growth factor (*EGF*), and carbachol (C).

treated cultured astrocytes. The induction levels by TPA across different genes varies considerably, suggesting the ERGs exhibit differential promoter control by TPA-activated pathways and/or oligodendrocytes can be heterogeneous with regard to PKC subtypes and hence respond differently to TPA treatment. Also note that EGF and carbachol induce high levels in some cells and that bFGF and PGDF are relatively weak inducers of ERGs in terms of amount of signal per cell and percentage of cells. It may be important that the addition of PDGF to bFGF-treated cultures actually results in a decreased level of expression in some ERG mRNAs.

ENCODING HELIX-LOOP-HELIX TRANSCRIPTION FACTORS

One of the most promising avenues of research into the genetic network of transcription factors that coordinate the spatial and temporal parameters of brain development has been the finding that helix-loop-helix proteins are expressed in the nervous system during development. The helix-loop-helix (HLH) (Table 35-3) motif is found in many proteins that act as either positive or negative regulators of gene expression and thus, exert remarkable control

TABLE 35-3. *HLH Transcription Factors in the Central Nervous System*

Class	Gene	Developmental Distribution
Class I: Basic-HLH		
IA	*E12/47*	Ubiquitous
IB	*MASH1*	Early neuronal
	MASH2	Early neuronal
IC	c-*myc*	ERG, ventricular, widespread
	L-*myc*	ERG, ventricular, early:wide, late:neuronal
	N-*myc*	ERG, ventricular, early:wide, late:glia
ID	*SCL*	Met/myelenceph ventricular zones
	NSCL1	Ventricular
	NSCL2	Ventricular
Class II: PROL-HLH		
	HES1 (hRHL)	ERG, widespread, ventricular
	HES3	Cerebellar Purkinje cells only
	HES5	Ventricular zone, retinal ganglion cells
Class III: HLH		
	Id1	Ventricular
	Id2	Cortex, cerebellum, neuronal?
	Id3 (HLH462, heir1)	ERG, ventricular, 1/α N-*myc* in neuroblastoma

FIG. 35-4. *In situ* hybridization examination of ERG expression in developing oligodendrocyte cultures. Secondary cultures of oligodendrocytes were cultured for several days on Labtek Culture slides. Cultures contain predominantly O4-positive cells. Cells were then either left untreated (C) or treated for 6 hours with either cycloheximide (*X*) or TPA plus X (*TX*) or bFGF plus X (*FX*). The message (NGF1BmRNA) is detected by standard *in situ* hybridization techniques. The amount of mRNA can be correlated with the amount of radiolabeled riboprobe specifically bound and is observed as the number of white grains present in the photographic emulsion above each cell. See text for details and Figure 35-5 for summary analysis of these results.

over cell phenotype and cell fate. All the members listed in Table 35-3 are found expressed in the developing and/or adult nervous system. The members can be categorized based upon the type of protein domain adjacent to the HLH domain. Class I possess a DNA-binding basic domain (a number of basic amino acids grouped together) adjacent to the HLH dimerization domain, hence called BASIC-HLH. BASIC-HLH proteins are the positive-acting promoter regulators recognizing the E-box, the DNA consensus sequence —CANNTG—. Included in this category are the ubiquitous E12 family of HLH proteins that are common partners of all the remaining HLH proteins. E12 heterodimers generally exhibit greater transcription transactivation than do homodimers of the members of the positive regulator families listed, such as MASH (mammalian aschaete-scute homologues) (Johnson et al., 1992), *myc* and SCL.

The other classes are distinguished by the modification of the basic amino acid domain that prevents homodimers or heterodimers from binding to the promoter elements: Class II (PROL-HLH) have a proline substitution in the basic domain and Class III (HLH) possess no basic DNA binding region at all. Heterodimerization of class I proteins with either class II or III inhibits the positive acting BASIC-HLH proteins by sequestering these active regulatory molecules into inactive pools. This binding of class II and class III to members of class I is now considered a major mode of transcription factor control over cell differentiation. The most fully delineated system of HLH control over cell fate is in muscle cell development.

Since these HLH genes directly participate in the control of cell lineage progression, and since HLH mRNAs have been described in the nervous system with a notable degree of differential cell distribution, we have begun to study the developmental expression patterns of these HLH genes in glial cells. Several of these genes, especially members of the ligand-inducible inhibitory classes II and III can be classified as ERGs and may be coordinately expressed along with other classes of ERGs by peptide signals. *In situ* hybridization analysis of cultured cells and tissue sections demonstrate many of these HLH genes are expressed in glial cells and hence may play a role in glial cell lineage (Arenander and de Vellis, unpublished data). The expression of classes II and III inhibitory HLH mRNAs (Arenander and de Vellis, unpublished data) suggests the existence of a positive acting HLH target in glial progenitor cells analogous to the MyoD story. It is also encouraging to find the system of interacting control responsible for modulating MyoD activity in myocytes may also function in glia as they progress through the stages of lineage development. The various extracellular ligands and the corresponding intracellular signaling pathways that modulate the key regulator of muscle progenitor fate, MyoD (Figure 35-6), are similar to those found to regulate the fate of glial cell progenitors during development (Arenander and de Vellis, 1994). Thus, we expect that an "*OligoD*" gene will be found expressed during oligodendrocyte cell development and that the expression of the various inhibitory HLH ERGs will exhibit an inverse correlation with the stages of glial differentiation.

CONTROL OF GLIAL-SPECIFIC LATE GENE EXPRESSION

We showed that GPDH is expressed in the central nervous system in a ligand-specific, developmental stage-specific, and cell-type-specific fashion (for review see Kumar and de Vellis, 1988). What elements control the transcription of GPDH? As suggested in the introduction, ligand-induced expression of ERGs are important candidates to regulate GPDH transcription. Northern analysis of both ERG and LRG message levels can display a temporal sequence suggesting a possible dependence between the two events. The hydrocortisone-mediated induction of gene expression in C6 glioma cells suggests a close correlation between the appearance of several ERGs and the LRG, GPDH (Cheng and de Vellis, unpublished data). However, simple correlation is not sufficient to determine causality. In particular, because of the combinatorial modes of ERG control, induction may not depend upon only one or two ERGs. In addition, many of the ERGs are expressed in a nonspecific manner (i.e., induced by many agents). In the case of GPDH, many of the same ERGs are expressed by activators of PKA (by forskolin (FOR) treatment leading to GPDH induction) and PKC (by TPA treatment, inhibiting GPDH induction).

Which of these rapidly induced transcription factors are important for the transcription of GPDH? The question we are asking must be extended to consider more than just the appearance of message as the critical parameter. Posttranslational modification of ERG proteins is well-known. If it is differentially controlled by PKC and PKA, which is likely, then the same ERGs can be induced by TPA and forskolin but their activity may be very different leading to either induction or inhibition of GPDH gene expression.

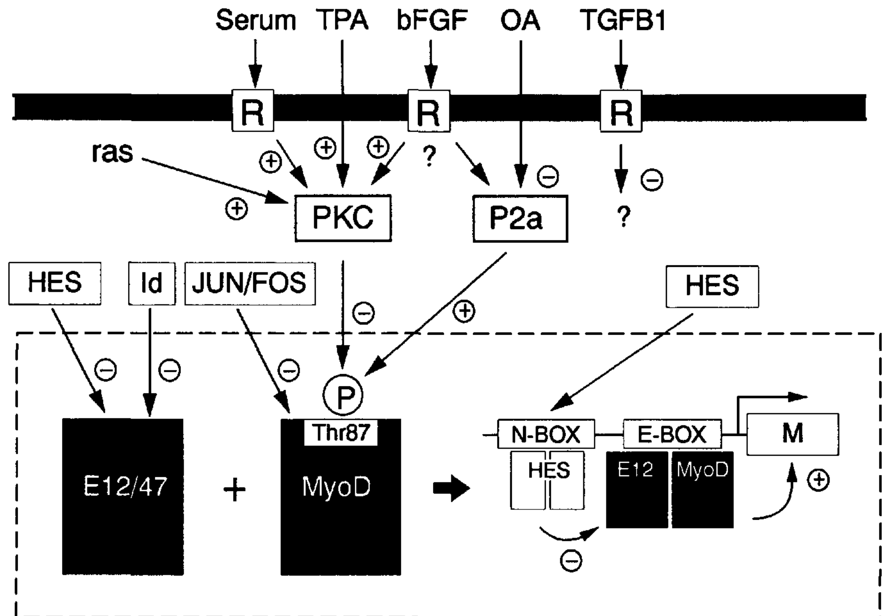

FIG. 35-6. Modulation of MyoD activity during muscle development. A number of agents control the development of myocytes. Note the analogy to oligodendrocyte developmental events. The agents in this figure all exert a negative influence on MyoD activity and thus inhibit myocyte differentiation. Note control is primarily exerted not at the level of MyoD expression, but rather at its activity as a positive regulator of muscle-specific gene expression (M in the gene box denotes muscle genes). Four levels of control are illustrated here. (*1*) HES dimers can bind to the N box in some muscle specific gene promoters which directly inhibits the activity of transactivating E-boxes. (*2*) HLH ERGs such as HES and Id can sequester E12 proteins into inactive pools. Lack of E12 proteins available for heterodimerization forces MyoD to form only homodimers, which are very weak transactivating factors of muscle-specific gene expression compared to heterodimers. The weakly active MyoD homodimers, however, are sufficient to continue to positively self-regulate MyoD gene expression. (*3*) The upregulation of JUN and FOS inhibit MyoD activity by directly binding to MyoD. (*4*) The threonine-87 residue in the basic region is a phosphorylation site that controls MyoD DNA binding activity. Agents shown converge on PKC, which then leads to phosphorylation of Thr-87. A parallel inhibition of phosphatase 2a (*P2a*), for example, by treatment with okadaic acid (*OA*), prevents dephosphorylation. The mode of inhibition of TGF β differs and remains unknown.

CONCLUSION: TRANSCRIPTION FACTOR NETWORKS FUNCTIONING IN GLIAL CELL DEVELOPMENT

Phenotypic progression of cells during development is by definition based on the expression of specific cellular proteins at each lineage stage. Since the expression of these proteins is a result of differential gene expression, the spectrum of transcription factors responsible for mediating the specific genomic programs represents a genetic control system or network (for review see Arenander and de Vellis, 1994). It is accepted that environmental agents control the fate of developing oligodendrocytes. Exposure of oligodendrocyte progenitor cells in culture to two of the main growth factors result in markedly different developmental end points (Figure 35-7). What is the difference in the genetic network that determines these different outcomes? It is possible that specific

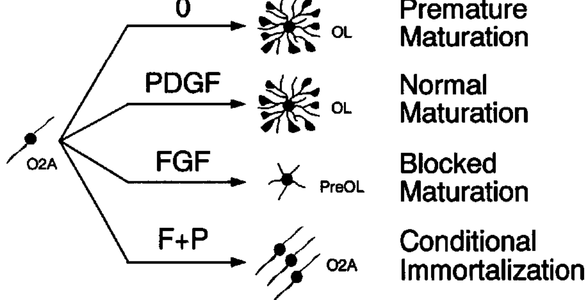

FIG. 35-7. Growth factor control of lineage decisions. Oligodendrocyte progenitor cells called oligodendroblasts (originally termed O-2A progenitor cells) will undergo four types of developmental processes depending upon the environmental signals present. Progenitor cells can become mature oligodendrocytes (*OL*) at two different rates, or enter and remain in a relatively undifferentiated stage (*Pre-OLs*) or continue indefinitely as early precursor cells.

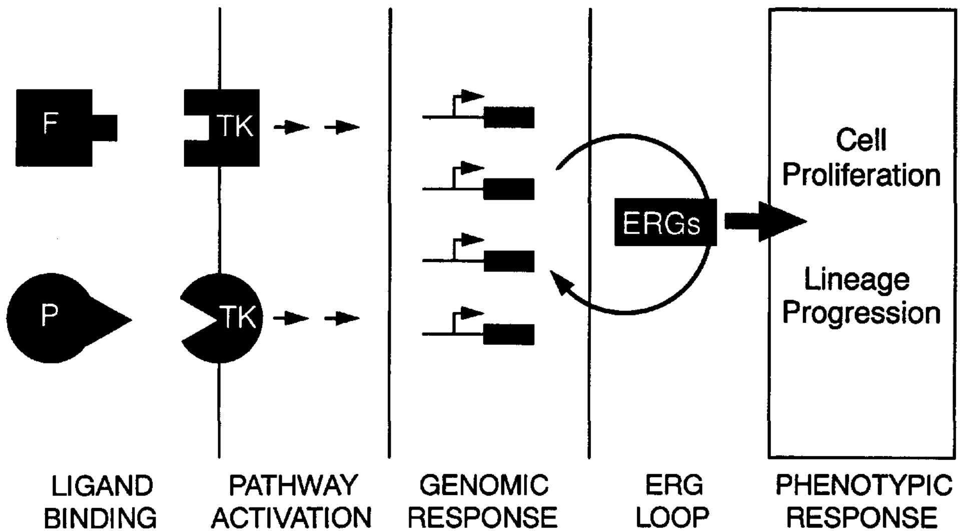

FIG. 35-8. ERGs mediating growth factor control of oligodendrocyte development. It is proposed that the differential expression of ERG encoding transcription factors in oligodendrocyte progenitor cells ("ERG loop") plays an important, possibly causal, role in determining cell response to extracellular signals (e.g., bFGF and/or PDGF) and hence glial cell fate during development.

FIG. 35-9. Transcriptional network control of glial cell lineage development. Four levels of transcription factor controls are depicted. Transcription factors can be responsible for different levels of control: (*1*) *lineage* (i.e., glia vs neuronal bifurcation), appearing in every early cell type), (*2*) *sublineage* (responsible for astrocyte vs oligodendrocyte decision), (*3*) *stage-specific* (responsible for stage progression and/or stabilization) and/or *process-specific* (i.e., proliferation) processes. In this hypothetical scheme, participation of transcription factors at each level of control is not mutually exclusive.

patterns of ERG expression play an important role in the network and contribute to the decision as to which genetic program to activate. Specifically, alternative patterns of growth factor-receptor coupling are encoded by differential ERG expression or "ERG-loops" as illustrated in Figure 35-8. These loops, in turn, coordinate the genetic expression required to carry out specific programs of proliferation and differentiation. Seen as a broader perspective, the transcriptional factor network composed of "constitutive" and induced proteins would assume different configurations depending upon the environmental signals that direct developmental decisions. Considering the whole of glial development, one can envision that present research efforts will eventually define the networks that encode for each major developmental decision. Toward that goal, we have outlined schematically alternative levels of network control over glial development (Figure 35-9).

Future research into the molecular mechanisms of ERG and LRG induction and their role in directing phenotypic development and cell function will benefit greatly from use of semiautomated injection into single cells of a side range of important reagents. Each cell in culture can now be manipulated as a complete *in vivo* system by injecting proteins, oligonucleotides, mRNA, DNA vectors, either individually or in combination and then examining the cell response over the period of minutes to days. Thus this approach provides a means to estimate the causal relationship of a particular process or molecule with a specific phenotypic response. Application of this technique permits more direct analysis than many current techniques that require either too much reagent or too many cells.

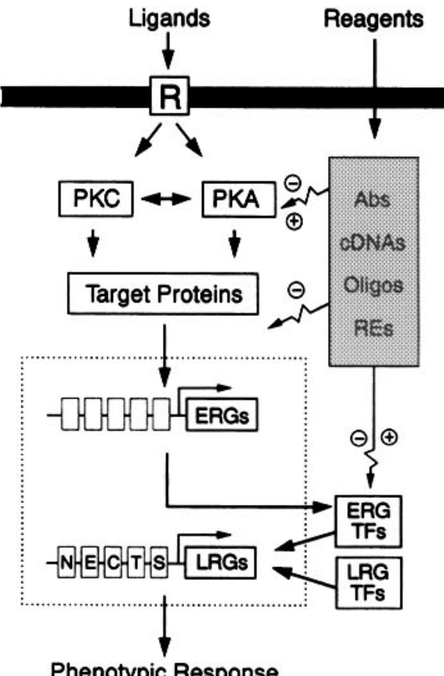

FIG. 35-10. Single-cell microinjection technology applied to the analysis of molecular mechanisms of glial development. The main signaling pathways leading to gene expression and phenotypic change are depicted. This technique permits injection into phenotypically defined cells of the glial lineage a variety of antibodies or antisense oligomers to block specific steps in the ligand-induced molecular cascade. One can also inject constitutive or inducible vectors to overexpress a specific RNA and/or protein to examine the influence on, for example, transcriptional control or to upregulate a particular step in the signaling cascade (i.e., kinase). TPs (Transcription factors). Dotted box is nucleus.

Microinjection modulation of ERGs will be valuable (see Figure 35-10). The work of R. Bravo and colleagues has demonstrated the potential inherent in the technique. Using highly purified reagents, they have been able to directly demonstrate the combinatorial control of ERGs over cell proliferation (Shuerman et al., 1989). Microinjection of antibodies with individual or pan specificity show the differential requirement for induction of each of the members of the *jun* and *fos* families in order to enable proliferation signals to function provides a model for investigating the causal mechanisms underlying similar processes during the various stages of glial lineage development. For example, injection of HLH vectors will permit upregulation of these key positively or negatively acting transcription factors and allow one to evaluated the predicted outcome in glial development. These data, in turn, can then be correlated with the results of *in vivo* distribution patterns of ERG expression during the development of the nervous system.

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36 Lipid metabolism

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The lipid composition and lipid metabolism of oligodendroglia (Morell and Toews, 1984; Pleasure et al., 1984) and astrocytes (Kanfer, 1986) have been comprehensively described, and therefore will not be reviewed here. The basic characteristics were largely established during the 1960s, 1970s, and 1980s. This investigative activity was undoubtedly due to the development of procedures for both the bulk isolation and the successful maintenance of primary cultures of these cell types. The current general interest in the functional significance of membrane phospholipase hydrolysis of membrane phospholipid initiated by receptor occupancy (Dennis et al., 1991; Exton et al., 1991) applies also to neuroglial cells.

SPHINGOGLYCOLIPIDS

Cerebrosides

Galactosylceramide and its sulfated derivative, sulfatide, are enriched in the myelin membrane and in the oligodendroglia cell that elaborates this membrane. Therefore, these two sphingoglycolipids have been extensively investigated in oligodendroglia cells. The enzyme catalyzing the synthesis of galactosylceramide, UDPgalactose:ceramide galactosyltranferase, is usually assayed *in vitro* with both nonhydroxyfatty acid (NFA) and hydroxyfatty acid (HFA) containing ceramides. The distribution of this catalytic activity was determined in subcellular particles prepared from bulk-isolated oligodendroglia of 18- to 19-day-old rats. The specific activity for the HFA ceramide galactosyltransferase activity was greatest in fractions containing Golgi and endoplasmic reticulum. The specific activity for the NFA galactosyltransferase was greatest in the fractions containing endoplasmic reticulum (Sato et al., 1988). This suggests independent sites for the synthesis and the existence of independent enzymes for the formation of HFA- and NFA-containing cerebrosides. The activity of UDPgalactose:ceramide galactosyltransferase correlated with the increasing number of cells staining with an antigalactosylceramide antibody of primary oligodendrocyte cultures (Neskovic et al., 1988). Adult rat brain slices were exposed to an antibody prepared to a presumed purified UDPgalactose:ceramide galactosyltransferase. The innermost and the outermost loops of the myelin sheaths, as well as the cytoplasm and processes of oligodendrocytes were positively immunostained (Roussel et al., 1987).

The incorporation of [^{3}H]galactose into cerebroside and sulfatide was examined during the growth of primary oligodendrocytes in culture. The degree of labeling increased from 15 to 29 days in culture and then gradually decreased. The increase in numbers of cells stained with antisulfatide and anticerebroside antibodies and the increased mass of sulfatide and cerebroside had similar developmental patterns, plateauing at 30 DIC. This is reminiscent of the *in vivo* temporal course of appearance of these markers (Singh and Pfeiffer, 1985). Exposure of primary cultures of oligodendrocytes from 21-day-old rat brain for 2 days to dibutyl cyclic AMP increased [^{3}H]galactose labeling of cerebroside 2.5-fold (Pleasure et al., 1986). The $^{35}SO_4$ incorporation into sulfatide and the level of cyclic nucleotide phosphodiesterase (CNPase) activity are very low in oligodendrocytes in serum-free medium devoid of triiodothyronine (T_3) as compared to cells maintained in the presence of T_3 (Koper et al., 1986). The effect of several agents on the incorporation of [^{3}H]galactose into lipids by bulk-isolated oligodendrocytes from bovine brain and 15- to 20-day-old rat brains or by primary rat brain oligodendrocyte cultures was investigated. The bulk-isolated rat brain oligodendrocytes and the oligodendrocyte cultures had 20 to 24% of the total labeled lipids present in cerebroside, and this was increased in cells exposed to hydrocortisone, T_3, ketone bodies, and retinoic acid. About 76% of the total radioactivity present in cerebrosides of the bovine oligodendrocytes was unaffected by added agents. It is difficult to determine the metabolic events in the cells affected by these agents and manipulations (Poduslo et al., 1990). Primary oligodendrocyte cultures were subjected to hypoxic conditions for 6 hours or to 12 nM oligomycin, and the appearance of labeled precursor in cerebrosides was measured: The incorporation of [^{3}H]palmitate into cerebrosides decreased by 50 to 60%, but the incor-

poration into ceramides increased. By contrast, hypoxia increased the incorporation of [^{3}H]galactose into cerebroside without affecting the activity of UDPgalactose:ceramide galactosyltransferase. It was speculated that hypoxia interferes with the transport of newly synthesized ceramide to the site of galactosylation, but it does not hinder the galactosylation of preexisting ceramide (Kendler and Dawson, 1990). Oligodendrocytes from lamb brain were maintained in suspension culture or allowed to adhere to polylysine-coated dishes under conditions favoring morphological differentiation. The incorporation of individual labeled precursors into cerebroside, sulfatide, neutral lipid, and ethanolamine plasmalogen was greater in the adhering cultures than in the suspension cultures. It appears that adherence to a substratum favors activation of systems for myelin membrane production (Vartanian et al., 1992).

Astrocyte cultures from the neopallium of mice were exposed to various labeled compounds. [^{3}H]Glycerol and [^{14}C]acetate were taken up equally and labeled all the major phospholipids; the latter was also found in cholesterol. [^{3}H]Galactose was present in cerebroside, but [^{3}H]SO_4 was not incorporated into sulfatides. These cultures may have contained a 4% oligodendrocyte contamination based on the determination of CNPase activity (Yim et al., 1986).

Gangliosides

The value for ganglioside oligodendrocyte bulk isolates in humans is 0.35 μg sialic acid/mg protein, in bovine, 0.52, and in 60-day-old rats, 1.45. Although there are species differences, the major gangliosides are GD_{1b}, GD_{1a}, GD_3, GM_1, and GM_3 (Yu et al., 1989). The ganglioside sialic acid content of bulk-isolated astrocytes from 20-day-old rats is 2.8 μg sialic acid/mg protein, which is greater than that of the oligodendrocytes or the neurons from similar aged animals. The ganglioside distribution of the astrocytes is 35% as GD_{1a}, 18% to 27% as GT_{1b}, 10% as GD_{1b}, and 12% as GM_1. As determined densitometrically, 11% is GM_3; however, with [^{3}H]glucosamine labeling this represents only 2% of the total as GM_3. These investigators found low levels of UDP-*N*-acetylgalactosamine:GM_3 *N*-acetylgalactosaminyltransferase activity, which is the enzyme catalyzing the synthesis of GM_2 (Byrne et al., 1988). The ganglioside content of primary cultures of rat brain astrocytes is 2.4 to 3.4 μg sialic acid/mg protein, which is similar to that present in the bulk-isolated astrocytes. GM_3 represented 71 to 81% of the total gangliosides present, which is distinctly different from the bulk isolates. The activity of UDP-*N*-acetylgalactosamine:GM_3 *N*-acetylgalactosaminyltransferase was barely detectable in these astrocyte cultures. This probably explains the high GM_3 content of the cells because the biosynthetic enzyme leading to the more complex ganglioside species is nearly undetectable (Sbasching-Agler et al., 1988).

NEUTRAL LIPIDS

Fatty Acids

Oligodendroglia convert acetoacetate and glucose to fatty acids and cholesterol to a greater extent than do astrocytes (Koper et al., 1984). C6 glioma cell cultures have the capacity for both elongation and desaturation of $C_{18:3(n-3)}$ to $C_{20:5(n-3)}$, $C_{22:5(n-3)}$, and $C_{22:6(n-3)}$ fatty acids. This is the established pathway for this metabolic route of fatty acid conversion (Cook and Spence, 1987; Cook et al., 1991). Primary astrocyte cultures convert $C_{18:2(n-6)}$ to $C_{20:4(n-6)}$ and $C_{18:3(n-3)}$ to $C_{20:5(n-3)}$, $C_{22:5(n-3)}$, and $C_{22:6(n-3)}$ fatty acids. In contrast, cultured neurons possessed only the Δ-4 desaturase activity, but not the chain elongation activity. Docosahexanoic acid ($C_{22:6(n-3)}$) is a major fatty acid present in the lipids of excitable membranes; these results suggest that the astrocytes biosynthesize this fatty acid and may release it for neuronal uptake (Moore et al., 1991). Confluent astrocyte cultures can convert palmitate and octanoate to CO_2 and acetoacetate. This acetoacetate formation is not through the mitochondrial HMG-CoA cycle but either by 3-keto acid CoA transferase, or by acetoacetate CoA deacylase, or by a combination of these two possibilities (Auestad et al., 1991). Primary rat brain neuroglia cultures have demonstrable monoacylglycerol and diacylglycerol lipase activity, which does not change with time in culture (Farooqui et al., 1990).

Cholesterol

Neurosteroids denote steroids detectable in brain tissue that are at levels independent of their plasma concentrations. Some are produced *de novo* in brain, while others are cholesterol metabolites. The neurosteroids bind to membrane receptors and exert no genomic influence. Some neurosteroids bind to the GABA receptors, and some inhibit or enhance excitatory amino acid receptor responses (Paul and Purdy, 1992). Mitochondria prepared from bulk-isolated oligodendroglia of 21-day-old rats convert [^{3}H]cholesterol to the steroids [^{3}H]pregnenolone and [^{3}H]pregnene-3β,20α-diol (Hu, 1987). Primary cultures of neonatal forebrain initially contain 60% oligodendrocytes and 40% astrocytes. The oligodendrocytes stain both with sep-

arate antibodies directed toward galactosylcerebroside and cytochrome $P450_{SCC}$, a cofactor for cholesterol side chain cleavage. The conversion of [^{3}H]mevalonate to cholesterol, pregnenolone, and pregn-5-ene-3β20α-diol correlate with the numbers of oligodendrocytes in these cultures. If a cytochrome P450 inhibitor is present under these conditions, only cholesterol is produced (Jung-Testas et al., 1989a, 1989b). Membranes of astrocytes and oligodendrocytes primary cultures were electrophoresed and the membranes from both cell types immunoblotted with anti-low density lipoprotein (LDL) receptor antibodies. [^{3}H]Cholesterol oleate-loaded LDL was converted to cholesterol and pregnenolone by these cultures (Jung-Testas et al., 1992).

The activities of both a microsomal and a myelin-associated form of cholesterol ester hydrolase increased by 20 days in oligodendrocyte cultures (Bhat and Pfeiffer, 1985). The content of free and esterified forms of cholesterol in C6 cells increased until the cultures are about 50% confluent subsequently, and declined as the cell number increased. There are parallel changes in both HMG-CoA reductase and acylCoA cholesterol acyltransferase activities. These correlate with the growth rate of cells and are not dependent upon LDL receptors or diffusible cytosolic factors (Friedman et al., 1987). Primary cultures of mixed brain cells were grown in media containing lipoprotein-free serum or medium containing lipoprotein-free serum supplemented with total lipoprotein or LDL or high-density lipoprotein (HDL). The conversion of acetate to sterol and HMG-CoA reductase activity were increased in the lipoprotein-free serum-grown cells as compared to the other cells. This suggests that cholesterol biosynthesis in neuroglia cells is under the usual control mechanisms (Shah, 1988). Transfer of C6 cells or primary astrocyte cultures from a 10% fetal calf serum medium to 10% lipoprotein-poor serum increases both sterol synthesis and HMG-CoA reductase activity. This is prevented if LDL is also present in combination with the lipoprotein-poor serum. HDL supplementation to the lipoprotein-poor serum further increases both sterol and HMG-CoA reductase activity of the primary astrocytes but not C6 cells (Langan et al., 1987). The content and magnitude of labeling of the cholesterol esters of cultured chick glia cells was increased when the cells were maintained in serum-free medium (Saito et al., 1987). C6 cells produced cholesterol esters by the biosynthetic enzyme lecithin:cholesterol acetyltransferase. The appearance of [^{14}C]oleate in cholesterol oleate was reduced in a dose-dependent manner by the presence of U1866A, a hypocholesteremic agent. This compound has no effect on lecithin:cholesterol acetyltransferase activity as measured *in vitro* (Jeng et al., 1985).

PHOSPHOLIPIDS

Content

Astrocytes were maintained in a defined serum-free medium for 2 weeks, and then the cells were placed in a serum-containing medium. The protein and DNA content were identical under both growth conditions. The cells maintained in a serum-free medium displayed typical fatty degeneration. The phospholipid content of the cells in a serum-containing medium increased markedly compared to that of cells in the serum-free medium (Krause and Debuch, 1987). Mitochondria, microsomes, and plasma membranes were isolated from primary astrocytes at 16, 19, and 24 days in culture, and the fatty acid composition of the phospholipids were determined. The principal fatty acid of all the phospholipids was palmitic acid. There were no differences in the fatty acids present in phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine of the plasma membrane fractions. Docosahexanoic acid was absent from the phospholipids of the plasma membrane, but was present principally in the phosphatidylethanolamines of the other membranes (El-Achkar et al., 1987). The total phospholipid content, expressed as picomoles phosphate per cell, was found to progressively increase in bulk-isolated oligodendroglia isolated from rats at 18, 30, 60, and 100 days of age. There were equivalent increases in all classes of phospholipids (Kreda et al., 1992). The plasmalogen content of the plasma membrane prepared from C6 cells is 2.5-fold greater than that of the endoplasmic reticulum, but the phosphatidylcholine content of the endoplasmic reticulum is 2.5-fold greater than that of the plasma membrane (Thomas et al., 1992).

Metabolism

C6 glioma cells, NN transformed astrocytes, and primary astrocyte cultures were exposed to [^{14}C]glycerol for 24 hours. Approximately 50 to 60% of the radioactivity was present in phosphatidylcholine, 20% in phosphatidylethanolamine, and 10% in phosphatidylserine-phosphatidylinositol. Similar labeling patterns were seen with [^{14}C]acetate and $H_3\,{}^{32}PO_4$. However, the cellular turnover rates of the phospholipids labeled by these precursors were not identical. There was a greater turnover of acetate-labeled lipids with the tumors, a greater turnover rate of [^{32}P] labeled lipids in primary cultures, but the turnover of glyc-

erol-labeled lipids was equivalent in the tumors and in the primary cultures (Hugues et al., 1985). There was an increased CNPase activity, a presumptive myelin marker, and characteristic morphological changes when C6 cells are grown in serum-free medium. This CNPase increase is not observed if the growth medium contains either monomethyl- or dimethylethanolamine, but does occur if either ethanolamine or choline is present in the medium. There are no differences in cell growth between these groups or in Na^+/K^+ ATPase activity. Phosphatidylmethylethanolamine, which is normally absent, represented 50% of the total phospholipid at the expense of phosphatidylcholine in cells grown in the presence of monomethylethanolamine. The K_{ms} of CNPase activity of untreated and treated cells was the same, but the V_{max} of the treated cells was decreased. This suggests that the polar head groups of the membrane phospholipids and the membrane fluidity may influence the normal myelination process (Volpe et al., 1986).

In a classical pulse-chase paradigm, choline-prelabeled C6 cells were chased with nonradioactive choline. During this chase period there was a 20-fold increase in free choline, a 1.7-fold increase in glycerophosphorylcholine, but little change in phosphorylcholine. This suggested a catabolic pathway of phosphatidylcholine → lysophosphatidylcholine → glycerophosphorylcholine → choline (Morash et al., 1988). The fatty acid composition of endogenous phosphatidylcholine and lysophosphatidylcholine was determined. The endogenous lysophosphatidylcholine composition resembled the product of phospholipase A_2 hydrolysis of endogenous phosphatidylcholine rather than that of phospholipase A_1 hydrolysis. When presented to cells, 2-acyllysophosphatidylcholine was converted to phosphatidylcholine more effectively than 1-acyllysophosphatidylcholine, suggesting that phospholipase A_2 was the initial enzyme initiating this catabolic pathway (Morash et al., 1989). Electropermeabilized C6 cells were employed to introduce labeled intermediates of the *de novo* pathway of phosphatidylcholine synthesis directly into the intracellular compartment. The label from [^{3}H]choline was found in both phosphorylcholine and phosphatidylcholine, but little was found in either glycerophosphorylcholine or cytidine diphosphate (CDP) choline. In contrast, neither phosphocholine nor CDP choline electropermeabilized into the cells labeled phosphatidylcholine. The radioactivity from the choline-labeled phosphatidylcholine was reduced by choline, but not by phosphocholine or CDP choline. This suggests that the individual enzymatic reactions of the *de novo* biosynthetic pathway are functionally linked, and that these reaction intermediates do not equilibrate with existing intracellular pools (George et al., 1989). This channeling of these intermediates is dependent upon the presence of intracellular Ca^{2+} (George et al., 1991a). The appearance of radioactive [^{3}H]choline, presented to C6 cell cultures, in phosphatidylcholine and choline, but not that present in phosphorylcholine, was increased threefold by the simultaneous presence of phorbol myristate acetate (PMA). This PMA response was not observed with ethanolamine labeling of phosphatidylethanolamine (Cook et al., 1989). Cytochalasin B, a filament-disrupting agent, but not colchicine, a microtubule depolymerizer, prevented the incorporation of both choline and ethanolamine into their corresponding phospholipids. This response appears to be specific for these two phospholipids, since $H_3{}^{32}PO_4$ incorporation into other phospholipids was unaltered. Similar alterations were not seen with other cytochalasins tested, suggesting that the reduction of phospholipid labeling is not a result of altered states of cellular microfilaments. It was proposed that cytochalasins causes Ca^{2+} release from intracellular stores (George et al., 1991b). Maximal incorporation of [^{3}H]serine, supplied to C-6 cell cultures, into phosphatidylserine occurs by 4 to 8 hours and into non-plasminylphosphatidylethanolamine by 12 to 24 hours. Approximately 80% of the radioactivity present in phosphatidylserine and phosphatidylethanolamine is retained in the head groups portion (Xu et al., 1991). This conversion of phosphatidylserine to phosphatidylcholine presumably is catalyzed by combined activities of the constitutive phosphatidylserine decarboxylase and phospholipid *N*-methyltransferases. The platelet activating factor (PAF) phosphocholine transferase and the lyso-PAF acetyltransferase activities are both present in cultured glial cells (Francesangeli et al., 1993).

The base exchange enzymes catalyze a replacement by serine, or by ethanolamine or by choline of the polar head groups of a preexisting phospholipid yielding the corresponding phospholipid (Kanfer, 1989). The developmental profile, the activities and subcellular distribution of the ethanolamine base exchange enzyme activity and the ethanolamine phosphotransferase activity of primary astrocyte cultures were found to be different (Mersel et al., 1987). The ethanolamine base exchange enzyme activity of spontaneously transformed astrocytes was lower than that of primary astrocyte cultures. There was a transient elevation of this activity by dibutyl cAMP treatment but not that of either the choline base exchange enzyme or ethanolamine phosphotransferase activities (El-Achkar et al., 1988). The sole mechanism available for the biosynthesis of phosphatidylserine in mammals is catalyzed by the serine base exchange enzyme (Kanfer, 1989). C6

cells were incubated with [^{14}C]serine in the presence or absence of Ca^{2+}, with several agents capable of modulating intracellular Ca^{2+}. Glutamate, A23187, and thapsigargin elevated the intracellular Ca^{2+} content and reduced the incorporation of serine into phosphatidylserine. It was proposed that the site of Ca^{2+} release from intracellular stores also corresponds to the location of the serine base exchange enzyme. This enzyme requires Ca^{2+} for activity and depletion of this cation might be expected to reduce the activity of this enzyme (Czarny et al., 1992).

Phospholipase A_2

The general experimental approach for these types of investigations are identical. Cells usually are prelabeled with arachidonate acid ($C_{22:4}$), which is incorporated into cellular phospholipids. The appearance of unesterified arachidonate in response to agonists is regarded as an index of cellular phospholipase A_2 activation.

C6 cells were exposed to muscimol, a selective GABA agonist, and there was an increase in unesterified $C_{22:4}$ with a small decrease in radioactivity present both in phosphatidylcholine and in phosphatidylethanolamine (Majewska and Chuang, 1985). Acetylcholine stimulated the appearance of $C_{22:4}$ when added to C6-2B cells, and this release was blunted by atropine. Carbachol, but neither oxotremorine or pilocarpine, had a similar effect, suggesting that occupancy of muscarinic receptors on these cells activated phospholipase A_2. The Ca^{2+} ionophore A23187 also increased the free $C_{22:4}$ content (DeGeorge et al., 1986a). Norepinephrine and other adrenergic agonists presented to primary rat brain astroglia cultures caused a liberation of $C_{22:4}$. A combination of norepinephrine, acetylcholine, and histamine resulted in a greater release of $C_{22:4}$ than either agent alone. This suggests that each agonist activated phospholipase A_2 by independent mechanisms (DeGeorge et al., 1986b). The acetylcholine-provoked release of $C_{22:4}$ by C6-2B glioma cells was blocked by quinacrine, a phospholipase A_2 inhibitor, and also by dexamethasone. This observation suggests that glucocorticoids may interfere with phospholipase A_2 activations (DeGeorge et al., 1987b). The acetylcholine-stimulated release of free $C_{22:4}$ by C6-2B cells does not occur in Ca^{2+} free medium or in the presence of Ca^{2+} channel blockers, suggesting that phospholipase A_2 activation is coupled to Ca^{2+} influx (Brooks et al., 1989). Similarly, the complement complex C5b-9 provoked the release of $C_{22:4}$ by ROC-1 cells, a fusion product of rat oligodendrocytes + C6 glioma cells, was Ca^{2+}-dependent and blocked by phospholipase A_2 inhibitors (Shirază et al., 1989).

Phosphatidylinositol-Specific Phospholipase C

A general experimental approach has been adopted by most investigators for demonstrating phosphopinositide specific phospholipase C activation. Cells usually are prelabeled with [^{3}H]inositol for 24 to 48 hours, the labeling medium replaced with a solution containing $LiCl_2$ and the agent to be tested. The appearance of water-soluble inositol phosphates is regarded as an index of inositol-containing phospholipid hydrolysis. Frequently these metabolites are separated by anion exchange chromatography into mono-, di-, and triphosphoinositols. This format of investigation was designed to determine if particular receptors are linked to phospholipase C. The metabolites produced are regarded as lipid-derived second messengers. Diacylglyceride is proposed to be a protein kinase C activator and inositol triphosphate is proposed to liberate Ca^{2+} from intracellular stores (Dennis et al., 1991).

Immunohistochemical examination employing antibodies to phospholipase C (PLC) isoforms was undertaken with primary rat brain cultures. Oligodendrocytes stain more intensely than astrocytes for PLCβ and PLCα, but astrocytes stained more intensely than oligodendrocytes for PLCδ (Mizuguchi et al., 1991).

Examples of agonist-provoked phospholipase C activity of astroglia and oligodendroglia are provided in Table 36-1.

Phospholipase D

Phospholipase D activity is of current interest because of a hope that it may contribute to metabolic control regulatory mechanisms (Dennis et al., 1991). Primary astrocytes were exposed to [^{3}H]glycerol to prelabel the phospholipid pool. Phorbol myristate acetate and dioctanoate, protein kinase C (PKC) activators, stimulated the phospholipase D-catalyzed appearance of the expected hydrolysis products, perhaps by a PKC-mediated mechanisms (Gustavsson and Hansson, 1990). The phosphatidylethanolamines of astrocyte primary cultures, 02A glia and C6 glioma cells were prelabeled with radioactive ethanolamine. The presence of either 10% fetal calf serum or phorbol myristate acetate resulted in the appearance of free ethanolamine in the media. This may have occurred by an activation of phospholipase D (McNulty et al., 1992).

[^{3}H]Choline-prelabeled 1321N1 astrocytoma cells

TABLE 36-1. *Receptor-Linked Phosphoinositide Hydrolysis*

Agent[a]	Cells	Reference
Astroglia		
Sphingosine	Primary astrocytes	Ritchie et al. (1992)
Psychosine	Primary astrocytes	Ritchie et al. (1992)
Tachykinin	Human U373MG astrocytoma	Lee et al. (1992)
Substance P	Primary astrocytes	Marriott and Wilkin (1992)
	Primary astrocytes from brainstem but not cerebral cortex	Beaujouan et al. (1990)
	Human astrocytoma UC113MG	Johnson and Johnson (1992)
Norepinephrine	Primary astroglia	Wilson et al. (1990)
Quisqualate	Primary astrocytes	Nicoletti et al. (1990)
Ibotinate	Primary astrocytes	Nicoletti et al. (1990)
Glutamate	Primary astrocytes	Nicoletti et al. (1990)
ATP, ADP	Primary astrocytes	Pearce et al. (1989)
Carbachol	Primary astrocytes	Pearce et al. (1988) Kastritis et al. (1992)
Norepinephrine	Primary astrocytes	Pearce et al. (1988) Ritchie et al. (1987) El-Etr et al. (1989)
Bradykinin	Primary astrocytes	Ritchie et al. (1987)
Carbamylcholine	Primary astrocytes from mesencephalons but not from cortex or striatum	El-Etr et al. (1989)
Prostaglandin F2α	Primary astrocytes	Kitanaka et al. (1991)
Histamine	Primary astrocytes	Arbones et al. (1988)
5-Hydroxytryptamine	Primary astrocytes	Hansson et al. (1987)
Oligodendroglia		
Chlorpromazine	C6	Leli et al. (1986) Leli et al. (1989)
Acetylcholine	C6-2B	Brooks et al. (1987) DeGeorge et al. (1987a)
Carbachol	Human oligodendroglioma	Post and Dawson (1992)
	C6	Ananth et al. (1987) Ritchie et al. (1987) Glanville et al. (1989)
Norepinephrine	C6	Ananth et al. (1987)
Dopamine	C6	Ananth et al. (1987)
Histamine	C6	Ananth et al. (1987) Glanville et al. (1989)
Serotonin	C6	Ananth et al. (1987) Glanville et al. (1989)
Endothelin	C6	Zhang et al. (1991)
Substance P	C6	Glanville et al. (1989)

[a]All the agents stimulated phosphoinositide hydrolysis.

were treated with carbachol, histamine, and bradykinin. These agents provoked the prompt release of phosphocholine into the culture medium, which preceded the release of choline. The intracellular source was not identified. In contrast, phorbol myristate acetate provoked choline release into the medium, but not the release of phosphocholine. Increased diacylglyceride was present in cells treated with either carbachol or phorbol myristate acetate. It was proposed that phosphatidylcholine hydrolysis by a phospholipase C was provoked by carbachol, but that phorbol myristate acetate provoked hydrolysis by phospholipase D (Martinson et al., 1989).

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VII Myelin

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37 Biochemistry of myelin proteins and enzymes

SAMMYE NEWMAN, MEGUMI SAITO, AND ROBERT K. YU

Myelin is formed as an extension of the oligodendrocyte (central nervous system) or Schwann cell (peripheral nervous system) plasma membrane. During myelination, glial cell processes contact and wrap around axons in a "jelly roll" pattern until a tightly compacted, multilamellar structure has formed around the axon. The compact myelin sheath is a continuation of the parent glial cell. However, the biochemical composition of myelin is distinct from that of the oligodendrocyte or Schwann cell plasma membrane from which it is derived (Szuchet et al., 1988).

Although structurally complex, myelin is biochemically quite simple in that it is comprised of relatively few major components. Lipids make up 70 to 85% of the dry weight of compact myelin, depending on the species, with the remainder of the myelin sheath consisting of proteins (15 to 30%) and gangliosides (~0.5%) (Rumsby, 1987; Morell et al., 1989). The proteolipid protein (PLP) and the myelin basic proteins (MBPs) together make up about 80% of the total protein content of compact central nervous system myelin (Morell et al., 1989). In the peripheral nervous system the P0 protein represents more than 50% of the total myelin proteins, while P2 and the MBPs constitute a significant portion (15 to 20%) of the remaining protein content. It is in the multitude of minor myelin proteins, splice products, and posttranslationally modified protein isoforms that the biochemical complexity of myelin is revealed. A number of structural proteins, enzymes, receptors, and second messenger-related proteins are now known to be integral to myelin and are regarded as essential for the normal molecular architecture and function of the compact myelin sheath. The list of myelin-specific and myelin-associated proteins continues to expand as techniques for their detection become increasingly sensitive. This chapter attempts to give the reader an appreciation of the myelin sheath as a complex and dynamic structure that is biochemically quite unlike any other. Proteins that are thought to function primarily in assembly and maintenance of the myelin sheath will be discussed, followed by those demonstrated to have enzymatic activity, receptor activity, association with second-messenger/signal-transduction pathways, or mitogenic activity. Readers unfamiliar with the morphology and molecular architecture of the myelin sheath are encouraged to consult any of several excellent monographs (Braun, 1977; Raine, 1977; Morell et al., 1989; Kirschner and Blaurock, 1992) for detailed treatment of these topics (see also Chapters 40, 41, and 42).

STRUCTURAL PROTEINS

The major proteins of myelin were initially thought to be primarily structural in nature, although recent evidence points to additional roles for these proteins. The major proteins in both central and peripheral nervous systems are myelin-specific, and almost all are known to exist as multiple isoforms derived by alternative splicing (see Chapter 38). These proteins tend to be strongly conserved among the higher vertebrate species, suggesting essential roles in the formation and maintenance of myelin. The distribution and relative abundance of some well-characterized myelin proteins are shown in Table 37-1. Figures 37-1 and 37-2 show the proposed orientation and localization of the major central and peripheral nervous system proteins in myelin.

Myelin Basic Proteins

The MBPs are a group of related protein isoforms derived from alternative splicing of a single gene. The structure and expression of the gene are now known to be considerably more complex than previously thought, resulting in a number of MBP and MBP-related products whose expression is developmentally regulated and not necessarily myelin-specific (see Chapter 38 for detailed discussion). In this chapter we focus on the well-characterized group of myelin-specific MBPs that arise from alternative splicing of exons 2, 5, and 6 of the classical MBP gene (for review see Campagnoni, 1988).

TABLE 37–1. *Distribution and Abundance of Major Myelin Proteins*

Protein	CNS	PNS	References
MBP	30	18	Greenfield et al. (1980)
PLP/DM20 family	50	<0.01[a]	Lees and Bizzozero et al. (1992) (CNS); Ikenaka et al. (1992) (PNS)
MAG	1	0.1	Quarles et al. (1992)
CNP	4	0.4	Tsukada and Kurihara (1992)
P0	<0.01	>50	Uyemura et al. (1992)
P2	<1	1–15	Martenson and Uyemura (1992) (CNS); Morell et al. (1989) (PNS)
PMP22/PASII	<0.01[a]	5–10	Snipes et al. (1992)
MOG	0.05	<0.01	Amiguet et al. (1992)

CNS, central nervous system; PNS, peripheral nervous system; MBP, myelin basic protein; PLP, proteolipid protein; MAG, myelin-associated glycoprotein; CNP, cyclic nucleotide phosphodiesterase; PMP22, peripheral myelin protein 22; MOG, myelin/oligodendrocyte glycoprotein.

Values correspond to the average percent of total myelin proteins in the adult mammalian nervous system. See text for localization of each component within myelin.

[a]mRNA encoding the specific protein has been detected, although neither the mRNA nor the protein is believed to exist in appreciable quantity in myelin.

As their name implies, the MBPs are a highly basic group of proteins, with isoelectric points above 10 (Smith, 1992). The diversity that results in this group from exon splicing is augmented by posttranslational modifications that sometimes confer charge heterogeneity. These modifications include loss of C-terminal arginine, *N*-acylation, glycosylation, phosphorylation, methylation, deamidation, and substitution of some arginine residues with citrulline (Toews and Morell, 1987; Morell et al., 1989; R. Smith, 1992). The MBPs are myelin-specific, extrinsic membrane proteins localized to the cytoplasmic

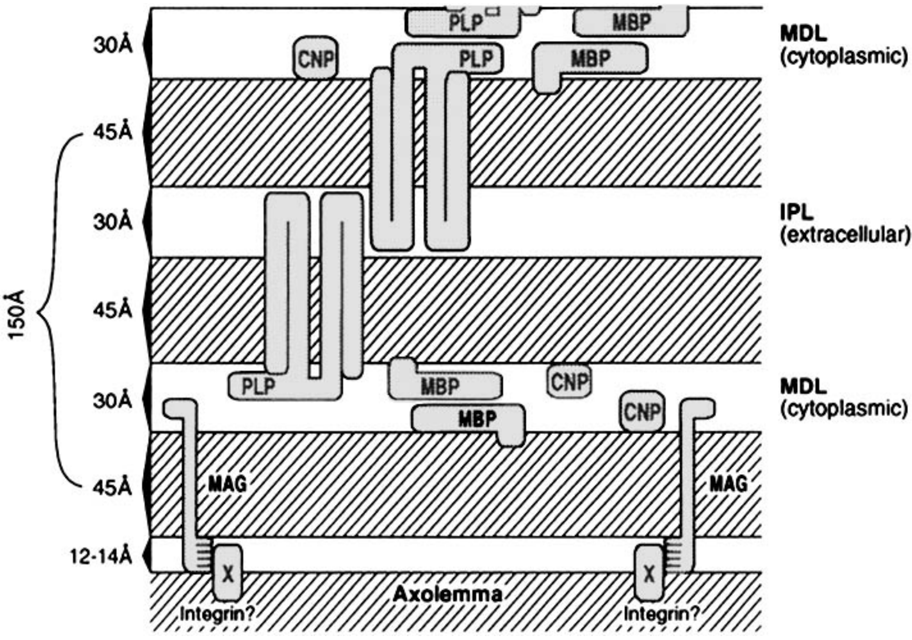

FIG. 37-1. Molecular architecture of central nervous system (CNS) myelin. The proposed orientation and localization of the major CNS myelin proteins are modeled after Lees and Bizzozero, 1992 (PLP); Smith, 1992 (MBP); Mendz, 1992 (MBP); Sprinkle, 1989 (CNP); Tsukada and Kurihara, 1992 (CNP); Salzer et al., 1990 (MAG); Trapp, 1990 (MAG); Quarles et al., 1992 (MAG). MAG is shown in the periaxonal myelin membrane, where it may mediate axon-glia adhesion through interaction with putative integrin receptor family molecules in the axolemma. CNP is concentrated in the inner mesaxon and periaxonal region of CNS myelin, as shown here, as well as in the paranodal compartments and noncompacted oligodendroglial ensheathments. The values for membrane spacing and periodicity are shown on the left and are averages for the CNS myelin of higher vertebrates (Kirschner and Blaurock, 1992). The *cross-hatched* sections represent the axolemma (lower section) or the extensions in the oligodendrocyte membrane (upper three sections). *MDL*, major dense line, *IPL*, intraperiod line; *PLP*, proteolipid protein; *MBP*, myelin basic protein; *CNP*, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; *MAG*, myelin-associated glycoprotein.

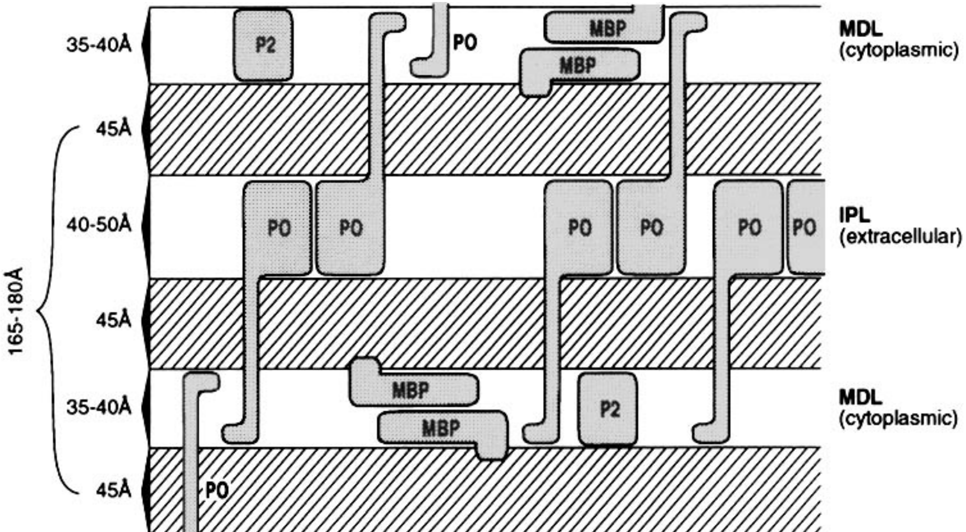

FIG. 37-2. Molecular architecture of peripheral nervous system (PNS) myelin. The proposed orientation and localization of the major PNS myelin proteins are modeled after Smith, 1992 (MBP); Mendz, 1992 (MBP); Kirschner and Blaurock, 1992 (P2 and P0); Colman et al., 1990 (P0); Filbin et al., 1990 (P0); Uyemura et al., 1992 (P0). MAG, not shown here due to its low abundance in PNS myelin, is thought to interact homotypically in the paranodal regions, mesaxon membranes, and Schmidt-Lantermann incisures in a manner similar to that presented here for P0 (see text). The values for membrane spacing and periodicity are shown on the left and are averages for the PNS myelin of higher vertebrates (Kirschner and Blaurock, 1992). The *cross-hatched* sections represent the extensions of the Schwann cell membrane. *MDL,* major dense line; *IPL,* intraperiod line; *MBP,* myelin basic protein.

membrane surface (major dense line) myelin of both the central and peripheral nervous system. The relative abundance of this class of proteins is greater in the central nervous system (~30% of rodent total myelin protein) than in the peripheral nervous system (~18% of rodent total myelin protein) (Greenfield et al., 1980). The abundance of MBPs in peripheral nervous system myelin is significant, suggesting an essential role for this class of proteins outside the central nervous system. However, data from dysmyelinating *shiverer* mutant mice would suggest otherwise. In the *shiverer* mouse, the majority of the MBP gene has been deleted, and MBPs are absent. Hypomyelination is the pathological hallmark of the *shiverer* central nervous system, with some myelin existing in an uncompacted state and lacking a major dense line (Readhead and Hood, 1990). In contrast, the peripheral nervous system of these mutants is relatively unaffected by the absence of MBP, implying that some other component(s), such as P0 or the basic P2 protein (see below), may substitute for MBP and be sufficient for formation of compact peripheral nervous system myelin. However, the relative abundance of MBPs in peripheral nervous system myelin implies a function that may be distinct from, and more subtle than, their role in the central nervous system.

Despite several decades of intense research, we still know relatively little about the precise structure of the MBPs in their native environment or the nature of their interactions with other biological molecules. Several recent reviews have discussed these topics and the problems inherent in their resolution in considerably more detail than can be presented here (Martenson, 1992; Mendz, 1992; Smith, 1992). MBPs appear to self-associate, and this association is promoted by lipids, implying that MBPs may exist as oligomers on the cytoplasmic surface of the myelin membrane (Smith, 1992). Up to one-third of the protein may penetrate between the lipid headgroups in the membrane (Smith, 1992; Mendz, 1992). The binding of a given MBP to an acidic lipid bilayer imposes more secondary structure on the protein than is seen in aqueous solution and also affects the organization of the lipid bilayer (Ong and Yu, 1984; Maggio and Yu, 1989, 1992; Mendz, 1992; Smith, 1992). There may be specific associations between MBPs and such acidic lipids as sulfatides and gangliosides, possibly resulting in stabilization and maintenance of the myelin structure (Ong and Yu, 1984; Maggio and Yu, 1989, 1992; Mendz, 1992). Biophysical studies support the idea that MBPs noncovalently cross-link apposed lipid bilayers in myelin and maintain the characteristic cytoplasmic spacing seen at the major dense line (Smith, 1992). Such studies have also provided clues regarding the possible significance of posttranslational modifications that introduce charge heterogeneity, such as phosphorylation, deamidation, and replacement of arginine residues with citrulline. The ability of the MBP

isomer to compact myelin is positively correlated with the net protein charge (Smith, 1992). Phosphorylation may serve to stabilize the functional ordered structure of the molecule, thus influencing its interaction with membrane lipids (Diebler et al., 1990).

Several models have been proposed for the secondary structure of MBPs (Stoner, 1984, 1990; Martenson, 1986, 1992). Historically, MBPs have not proven amenable to spectroscopic analyses or crystallization for x-ray diffraction studies. In fact, a recent study by Sedzik and Kirschner (1992) indicated that MBP does not possess any crystalline structure. Nonetheless, it has been possible to make predictions about conformation of the native molecule using algorithms that consider primary amino acid and cDNA sequences, the polycationic nature of the posttranslationally modified isomers, and evolutionary conservation of sequences and intron-exon structure (Stoner, 1984, 1990; Martenson, 1986, 1992). Although the details may vary somewhat, these models generally evoke a β-structure in which exon-intron junctions occur near the ends of predicted β-strands that are interrupted by large and small loops (Stoner, 1990). Hydrophobic residues in the large loop regions may mediate MBP insertion between the lipid head groups of the membrane bilayer (Martenson, 1992), while MBP self-association may contribute to myelin spiraling (Stoner, 1990).

Proteolipid Protein and DM20

The PLP is the most abundant protein in central nervous system myelin—about 50% in adult animals—and is absent from peripheral nervous system myelin. PLP is an integral membrane protein with several transmembrane domains. It has a high isoelectric point (>9) and a high content of cysteine residues, both as free thiols and as disulfide bonds (Lees and Bizzozero, 1992). Like many other myelin-specific proteins, the proteolipid proteins of myelin represent a class of proteins that are the products of alternative splicing of a single gene. The predominant isoform has an apparent molecular weight on SDS (sodium dodecyl sulfate) polyacrylamide gels of 25 kD and is commonly referred to as PLP. A second isoform, DM20, migrates during SDS polyacrylamide gel electrophoresis (SDS-PAGE) as a 20 kD band. Resolution of the primary sequences of PLP and DM20 has shown their authentic molecular weights to be 30 kD and 26 kD, respectively (Lees and Bizzozero, 1992; Macklin, 1992). DM20 is identical to PLP except for the deletion of amino acid residues 116–150 (Macklin, 1992). The high abundance of PLP in the myelin membrane and the striking evolutionary conservation of its amino acid sequence point to a critical role in the structure and function of central nervous system myelin.

PLP and DM20 are both acylated, containing covalently linked palmitic, oleic, and stearic acids as the major acyl groups (Toews and Morell, 1987; Lees and Bizzozero, 1992). Acylation appears to be a late posttranslational modification that takes place within myelin at a rapid turnover rate and requires fatty acyl-CoA as the acyl chain donor. The reaction occurs by an autocatalytic process, or autoacylation (Lees and Bizzozero, 1992). Deacylation is directed by a separate, myelin-associated protein fatty acylesterase (Bizzozero et al., 1992). The high content of acyl chains (2 to 2.4% by weight) may contribute to the interaction of PLP with the lipid bilayer of myelin (Toews and Morell, 1987).

The orientation of PLP in the myelin membrane has been examined using chemical and immunological approaches, but the data remain inconclusive. Different topographical models postulate from 2 to 4 membrane-spanning domains with extramembranous domains situated in both the cytoplasmic and extracellular spaces (Stoffel et al., 1984; Hudson et al., 1989; Lees and Bizzozero, 1992). The extracellular domains may be instrumental in stabilizing the intraperiod line of myelin (Morell et al., 1989). Functional studies using reconstituted membrane systems support an active role for PLP as an ion channel (Toews and Morell, 1987; Lees and Bizzozero, 1992). Clarification of the exact conformation of the protein in the intact myelin sheath will no doubt assist in defining its function.

DM20 is a relatively minor product of the PLP gene. However, recent studies have uncovered an intriguing pattern of DM20 developmental expression and tissue specificity. DM20 is expressed earlier in development than PLP and is in fact the major PLP gene product in the developing embryo (Macklin, 1992; Ikenaka et al., 1992; Timsit et al., 1992). Although neither gene product is readily detectable in peripheral nervous system myelin, both PLP and DM20 appear to be expressed in very low levels in Schwann cells in the peripheral nervous system (Puckett et al., 1987; Ikenaka et al., 1992). DM20 expression has been demonstrated in nonglial cell types as well (Campagnoni et al., 1992; Ikenaka et al., 1992). The appearance of DM20 in "premyelinating" glial cells and in cells outside the glial cell lineage suggests that this protein has a function(s) unrelated to myelination. Data from studies using dysmyelinating mutant animals point to a unique role for DM20 in glial cell differentiation (Macklin, 1992; Schneider et al., 1992). Almost all mutations identified in the PLP gene thus far are characterized

by hypomyelination as well as by oligodendrocyte degeneration and death (Hudson, 1990; Macklin, 1992). There is, however, a point mutation in the PLP gene of *rumpshaker* mice that results in hypomyelination without glial cell death (Schneider et al., 1992). Although sparse, some myelin sheaths subsist in the *rumpshaker* mutants and these showed selective immunostaining for DM20 using antibodies specific for PLP alone or PLP plus DM20 (Schneider et al., 1992). These findings suggest that DM20 may serve a critical purpose in glial cell development that is distinct from any function in myelin formation and maintenance.

Myelin-Associated Glycoproteins

The myelin-associated glycoproteins (MAGs) are heavily glycosylated and are specific to myelin sheaths. Although this class of proteins is relatively minor (1% of central nervous system/0.1% of peripheral nervous system myelin proteins) as compared to the MBP and PLP gene products, MAG is highly concentrated in the periaxonal regions of both central and peripheral nervous system myelin. In the peripheral nervous system, MAG immunostaining is also seen in glial membranes of the Schmidt-Lantermann incisures, paranodal loops, and mesaxons (Trapp, 1990). MAG is not seen within the compact myelin sheath in either the central nervous system or peripheral nervous system. This distribution pattern is consistent with the postulated role of MAG in membrane-membrane interactions during myelin formation and maintenance (Morell et al., 1989; Salzer et al., 1990; Quarles et al., 1992).

MAG derived from the central nervous system migrates as a single broad band of ~100 kD during SDS-PAGE. Enzymatic deglycosylation of the sample resolves this band into two equally intense bands with apparent molecular weights of 72 and 67 kD (Salzer et al., 1990; Quarles et al., 1992). Biochemical and molecular studies have revealed that the latter two bands represent two isoforms of MAG (L-MAG and S-MAG, respectively) that are derived by alternative splicing from a single gene. The MAGs have a carbohydrate content of ~30% (Quarles et al., 1992), with eight potential *N*-linked glycosylation sites predicted by the cDNA structure (Trapp, 1990). The complete structures of the carbohydrate side chains are unknown, but it is believed that they possess the HNK-1/L1 (or sulfated glucuronic acid [SGA]) epitope present in many cell adhesion molecules. The primary sequences of the MAGs predict a single transmembrane domain, a large extracellular domain that contains five IgG-like disulfide loop regions and the eight glycosylation sites, and C-terminal cytoplasmic domains with potential phosphorylation sites (Trapp, 1990; Quarles et al., 1992). The C-termini of L-MAG and S-MAG differ as a result of an earlier translation termination codon in S-MAG mRNA (Quarles et al., 1992). L-MAG is produced almost exclusively in the central nervous system, and is the predominant variant during early development and active myelination (Campagnoni, 1988; Trapp, 1990). S-MAG is the major isoform in the adult central nervous system and in the peripheral nervous system at all ages. Differences in phosphorylation and other posttranslational modification may be responsible for the differences observed in MAG distribution in central nervous system and peripheral nervous system, perhaps by mediating interaction with the cytoskeleton (Quarles et al., 1992; Trapp, 1990). Other posttranslational modifications of the MAGs include sulfation of oligosaccharide moieties and acylation of the transmembrane domain (Quarles et al., 1992).

The MAGs are members of the immunoglobulin superfamily by virtue of the extracellular immunoglobulin domains seen in both isoforms. Other members of the immunoglobulin superfamily include nervous system molecules that are involved in cell-cell adhesion, such as neural cell adhesion molecule (N-CAM) (Salzer et al., 1990; Trapp, 1990; Quarles et al., 1992). Another feature of the extracellular domain that is common to both L-MAG and S-MAG is the tripeptide sequence Arg-Gly-Asp (RGD) found in other cell recognition molecules as part of a binding site for the integrin receptor family (Salzer et al., 1990). All membranes that are enriched in MAG appose other membranes by a space of 12 to 14 nm (Trapp, 1990). The structure and localization of MAG thus strongly connote possible function in cell adhesion and membrane spacing (Trapp, 1990). Homotypic interaction may be operational in Schmidt-Lantermann incisures, paranodal loops, and mesaxon membranes in peripheral nervous system myelin, while heterotypic interactions with axolemmal constituents may mediate glia-axon adhesion (Salzer et al., 1990; Trapp, 1990; Quarles et al., 1992).

P0 Protein

The 28 kD P0 glycoprotein accounts for more than 50% of myelin protein in the peripheral nervous system, where it is an integral membrane protein expressed exclusively by myelinating Schwann cells (Kitamura et al., 1976; Uyemura and Kitamura,

1991; Uyemura et al., 1992). The location and abundance of P0 in peripheral nervous system myelin have led to speculation that P0 is the counterpart of PLP, even though the biochemical and physical characteristics of these two proteins are quite different. In contrast to other major myelin proteins, only one transcription product has been identified for the P0 gene.

Like MAG, P0 belongs to the immunoglobulin superfamily and is thought to play a role in adhesion between adjacent membranes. The predicted orientation of P0 in the myelin membrane resembles that postulated for MAG: a positively charged cytoplasmic C terminus is followed by a transmembrane domain and an extracellular glycosylated N-terminal region that bears a single immunoglobulinlike domain with a characteristic intradomain disulfide bond (Uyemura et al., 1992). In addition to glycosylation, the P0 protein undergoes phosphorylation, sulfation, and acylation (Uyemura and Kitamura, 1991; Uyemura et al., 1992). Unlike MAG, the P0 protein does not contain an RGD sequence and is found in compact myelin rather than localized to the axon-glia interface. These features point to a role for P0 in homotypic interactions across the intraperiod line.

Research using transfected cells that express P0 protein supports the idea that P0 serves as a homophilic adhesion molecule (Colman et al., 1990; Filbin et al., 1990). In this model, P0 molecules in adjacent layers of myelin would interact to bridge the extracellular space and form the intraperiod line. On the cytoplasmic membrane surface, the positively charged intracellular domain may interact with acidic membrane lipids to help form the major dense line (Uyemura and Kitamura, 1991; Uyemura et al., 1992). Recently, two groups independently demonstrated that mutation in the human P0 gene is associated with Charcot-Marie Tooth disease type 1B, an inherited peripheral nervous system neuropathy (Hayasaka et al., 1993; Kulkens et al., 1993).

P2 Protein

The P2 protein is a basic 14 kD peripheral membrane protein that is enriched in peripheral nervous system relative to central nervous system myelin (Uyemura and Kitamura, 1991). Despite the superficial similarity to the MBPs, there is no primary sequence homology between P2 and MBPs (Uyemura and Kitamura, 1991; Narayanan et al., 1988). However, the abundance of MBPs and P2 in peripheral nervous system myelin seem to be interdependent, in that the peripheral nervous system content of each protein varies among mammalian species, but the cumulative content of the MBPs plus P2 is relatively constant (Uyemura and Kitamura, 1991). This reciprocal relationship implies that the MBPs and P2 may be functionally related. P2 content ranges from less than 1% of peripheral nervous system myelin protein in rats and mice to approximately 15% of bovine peripheral nervous system myelin protein (Morell et al., 1989). Like the MBPs, P2 is localized to the cytoplasmic side of the myelin membrane and is thus a good candidate for a role in formation and maintenance of the major dense line (Morell et al., 1989; Uyemura and Kitamura, 1991; Martenson and Uyemura, 1992). There is, in fact, a direct correlation between the P2 content and the width of the cytoplasmic space in peripheral nervous system myelin in different species (Kirschner and Blaurock, 1992). The phylogenetic conservation of P2 is slightly higher than that of the MBPs (Uyemura and Kitamura, 1991; Martenson and Uyemura, 1992).

A distinct physiological role for P2 is suggested by its striking homology with the family of lipid-binding proteins that includes cellular retinoic acid-binding protein, cellular retinol-binding protein, and fatty acid-binding protein (Narayanan et al., 1988; Martenson and Uyemura, 1992). Indeed, high-affinity binding of bovine P2 with oleic acid, retinoic acid, and retinol has been observed (Uyemura et al., 1984). Thus, the P2 protein may be involved in solubilization and/or transport of fatty acids in myelin (Martenson and Uyemura, 1992).

PMP22/PASII

Kitamura and coworkers (1976) isolated an acid-insoluble peripheral nervous system-specific glycoprotein designated PASII, based on its electrophoretic properties and staining with periodic acid-Schiff reagent. The bovine PASII was characterized as an integral membrane protein containing glucosamine, mannose, galactose, fucose, and glucose and was further observed in peripheral nerve myelin from a number of species (Kitamura et al., 1976). More than a decade later, differential screening of cDNA libraries prepared from the distal stump of a crushed sciatic nerve and the normal contralateral nerve led to identification of a rat mRNA that is repressed in the injured nerve stump but present at high levels in naive rat sciatic nerve (De Leon et al., 1991). The protein encoded by this mRNA, designated PMP22 (peripheral myelin protein 22), is present in compact peripheral nervous system myelin as ~5 to 10% of total myelin protein (Snipes et al., 1992) and has been implicated in growth arrest (Welcher et al.,

1991; Suter et al., 1992). PMP22 and PASII are now known to be identical (Welcher et al., 1991; Snipes et al., 1992). The PMP22 mRNA sequence predicts an 18 kD protein with an *N*-linked glycosylation site that accounts for the observed molecular mass of 22 kD (Welcher et al., 1991). Although the mRNA has been detected in brain, no PMP22 protein has been observed in the central nervous system (Snipes et al., 1992). Mutation in the gene encoding this protein is responsible for the *trembler* mutation in mouse, which is characterized by severe hypomyelination and continuing Schwann cell proliferation throughout life (Suter et al., 1992). Mutation in the PMP22/PASII gene is also implicated in Charcot-Marie-Tooth disease type 1A (not to be confused with CMT type 1B, an apparent P0 protein-associated defect; see above) (Valentijn et al., 1992a, 1992b).

Myelin/Oligodendrocyte Glycoprotein

Myelin/oligodendrocyte glycoprotein (MOG) is a central nervous system-specific glycoprotein (Linington et al., 1984) representing ~0.05% of myelin proteins (Amiguet et al., 1992). SDS-PAGE of purified MOG reveals a doublet at 26 to 28 kD and a minor band at ~51 kD. Enzymatic deglycosylation results in a single band at ~25 kD, indicating that the 26 and 28 kD proteins represent differentially glycosylated products that may form dimers (Amiguet et al., 1992). Immunocytochemistry has localized MOG to the extracellular surface of myelin sheaths and oligodendrocytes (Brunner et al., 1989). The distribution pattern and the observation that MOG appears late in development relative to other myelin proteins (Matthieu and Amiguet, 1990) suggest that it might signal cessation of further myelination. The primary sequence of the rat mRNA encoding MOG has been determined (Gardinier et al., 1992). The deduced amino acid sequence places MOG in the immunoglobulin superfamily of proteins and predicts two transmembrane domains, unique among the immunoglobulin superfamily members, with a single immunoglobulinlike domain and *N*-linked glycosylation site on the extracellular membrane surface (Gardinier et al., 1992). MOG displays significant sequence homology with bovine butyrophilin, an acidic membrane glycoprotein that may serve as a lipid receptor. The sequence analysis thus connotes a potential recognition role for MOG in lipid interactions in myelin (Gardinier et al., 1992).

Myelin/Oligodendrocyte-Specific Protein

Like MOG and PLP, myelin/oligodendrocyte-specific protein (MOSP) is an integral membrane protein that is specific to central nervous system myelin. Monoclonal antibody studies show that MOSP, a 48 kD protein with a pI of 6.7, is abundantly expressed on the oligodendrocyte cell surface (Dyer et al., 1991). Moreover, specific antibody binding to cultured oligodendrocyte membrane sheets causes MOSP to redistribute directly over cytoplasmic microtubules to which cyclic nucleotide phosphodiesterase is colocalized (Dyer and Matthieu, 1993). MOSP is capable of transducing signals that result in an increase in microtubular structures within oligodendrocytes; this is the only surface membrane component known to date that has these signaling capabilities in oligodendrocytes (Dyer, 1993). Thus, MOSP may be critical to membrane-cytoskeleton interactions during assembly and maintenance of myelin (Dyer et al., 1991).

MYELIN-ASSOCIATED ENZYMES

In the past, myelin was believed to be metabolically inert and to function mainly as an electrical insulator that plays an important role in facilitating nerve conduction. This concept was derived from metabolic studies showing that myelin was the most stable component among brain subcellular fractions, and was consistent with initial reports demonstrating that isolated myelin preparations contained no enzyme activity except leucine aminopeptidase (Adams et al., 1966). However, since the discovery of 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) in purified myelin preparations (Kurihara and Tsukada, 1967), much attention has been directed toward enzymes intrinsic to myelin and their physiological significance. To date, the existence of more than 40 different enzyme activities in central nervous system myelin has been reported. The finding of this vast array of myelin-associated enzymes has forged the concept that myelin is not only an electrical insulator, but also may carry out active metabolism with its intrinsic enzyme activities. Indeed, it has been shown that lipid and protein components in myelin have measurable turnover rates and are subjected to metabolic turnover even after the period of active myelination (Smith, 1968; Horrocks, 1969; Benjamins and Smith, 1977; Miller et al., 1977; Ando et al., 1984). Recent studies have demonstrated that myelin membranes possess receptor and signal transduction activities, including high-affinity muscarinic cholinergic receptors (Larocca et al., 1987a) and G proteins (Bernier et al., 1989; Boulias and Moscarello, 1989; Braun et al., 1990b; Larocca et al., 1990, 1991), in addition to related enzyme activities such as adenyl cyclase, phospholipases, and enzymes involved in phosphoinositide

metabolism (see below). Evidence for the possible linkage between these receptors and formation of second messengers in myelin has also been presented. For example, treatment of myelin with effectors for cholinergic receptors caused the hydrolysis of phosphoinositide-4,5-bisphosphate (PIP_2) (Larocca et al., 1988) or G proteins (Golly et al., 1990). These findings imply that myelin membranes may respond to neurotransmitter and/or hormonal signals from the environment.

Table 37-2 summarizes the myelin-associated enzymes reported to date. In this section the localization, properties, developmental profiles, and physiological significance of these enzymes will be discussed. For further information, the reader is referred to several excellent reviews (Suzuki, 1980; Norton, 1981; Norton and Cammer, 1984; Ledeen, 1992).

2′,3′-Cyclic Nucleotide 3′-Phosphodiesterase

CNP is a myelin-associated enzyme that hydrolyzes 2′,3′-cyclic nucleotides to produce exclusively 2′-nucleotides (Sprinkle, 1989; Tsukada and Kurihara, 1992). However, no physiologically relevant substrate for CNP has yet been found in myelin, despite many frustrating searches. The current view is that the enzymatic activity may be vestigial and unrelated to the functional significance of the molecule in myelin (Braun et al., 1990a; Tsukada and Kurihara, 1992). For this reason, CNP may be considered with those myelin proteins thought to play primarily an architectural role, with the caveat that the authentic function of CNP in myelin is as yet unknown.

CNP is the principal component of the Wolfgram protein fraction of myelin, comprising ~4% of central nervous system myelin proteins. The enzyme is unique to oligodendrocytes in the central nervous system, but is found in peripheral nervous system myelin in lower abundance (~0.4%) and in other cell types outside the nervous system at very low levels of activity (Vogel and Thompson, 1988; Sprinkle, 1989; Tsukada and Kurihara, 1992). SDS-PAGE of purified CNP under reducing conditions resolves two bands with apparent molecular weights between 43 and 48 kD, depending on the species studied (Müller et al., 1981; Müller, 1982; Tsukada and Kurihara, 1992). These isoforms have been designated CNP1 and CNP2, respectively. The enzyme is posttranslationally modified by phosphorylation and by acylation of the N-terminal serine residue (Sprinkle, 1989). Molecular cloning studies have revealed that two rodent CNP mRNAs, differing in their 5′ ends, are derived by alternative splicing (Kurihara et al., 1990; 1992). CNP appears early in development, prior to myelination (Braun et al., 1990a), and is maintained at high levels throughout myelin formation and maintenance (Vogel and Thompson, 1988; Tsukada and Kurihara, 1992). Most studies suggest a cytoplasmic localization for CNP (Vogel and Thompson, 1988). Although CNP copurifies with myelin membranes, it is not observed in compact myelin (Sprinkle, 1989; Braun et al., 1990a; Tsukada and Kurihara, 1992). Instead, it is highly concentrated in the paranodal compartments, periaxonal membrane, inner mesaxon, and noncompacted oligodendroglial ensheathements (Sprinkle, 1989; Braun et al., 1990a). Although interspecies variation in CNP sequence is relatively low, there seems to be more evolutionary tolerance for mutation in CNP than in MBP or PLP (Tsukada and Kurihara, 1992).

A number of putative functions have been proposed for CNP, including a role in tRNA ligase reactions, cell adhesion, or kinase reactions (reviewed by Vogel and Thompson, 1988; Sprinkle, 1989; Tsukada and Kurihara, 1992). CNP has been shown to colocalize with cytoskeletal elements (Dyer and Benjamins, 1989; Braun et al., 1990a) and appears to self-associate in dimers or perhaps higher order oligomers (Vogel and Thompson, 1988; Braun et al., 1990a). These features are consistent with the notion that CNP may be a key component in an interactive protein network within the glial cell. A compelling line of evidence for a putative CNP function involves isoprenylation of CNP1 at a C-terminal cys-X-X-X-COOH site (Braun et al., 1991). Transfection studies have been performed using nonmyelinating mammalian cells and cDNAs encoding either wild-type CNP or a cys→ser mutant of CNP that cannot be isoprenylated. These studies have shown that only the wild-type protein causes these cells to extend filopodia and processes. Moreover, only the wild-type protein is localized to the cell membrane, whereas the cys→ser mutant CNP remains cytoplasmic (Gravel et al., 1993). These intriguing data suggest a possible role for CNP in oligodendrocyte process extension. Other proteins known to be isoprenylated at the C-terminus, such as *ras* proteins and some G protein subunits, are associated with signal transduction pathways (Braun et al., 1991). The presence in CNP of potential nucleotide-binding domains (Sprinkle, 1989) further strengthens the hypothesis that CNP may exert a regulatory influence on cellular events such as growth and differentiation by serving as part of a bridge between extracellular signals and intracellular effector molecules.

Cholesterol Ester Hydrolase

Eto and Suzuki (1973a) first reported that rat brain contained three different cholesterol ester hydrolase

TABLE 37–2. *Myelin-Associated Enzymes*

Enzyme	Reference
Unknown substrate	
2′,3′-Cyclic nucleotide-3-phosphodiesterase (CNPase)	Kurihara and Tsukada (1967); Sims and Carnegie (1978); Sprinkle et al. (1978, 1989); Wells and Sprinkle (1981); Vogel and Thompson (1988); Trapp et al. (1988); Agrawal et al. (1990)
Sterol metabolism	
Cholesterol ester hydrolase (cholesterol esterase)	Eto and Suzuki (1971, 1973a, 1973b); Johnson and Shah (1986)
Cholesterol ester synthetase (cholesterol acyltransferase)	Choi and Suzuki (1978); Jagannatha and Sastry (1981)
Testosterone 5α-reductase	Melcangi et al. (1988)
Glycolipid (or glycoprotein metabolism)	
UDP-galactose:ceramide galactosyltransferase	Nescovic et al. (1973); Costantino-Ceccarini and Suzuki (1975); Koul et al. (1980, 1981); Roussel et al. (1987)
Neuraminidase (sialidase)	Yohe et al. (1983, 1986); Saito and Yu (1986, 1992a, 1992b)
Phospholipid-synthesis	
Ethanolamine kinase	Kunishita et al. (1987b)
CTP:phosphoethanolaminecytidyltransferase	Kunishita and Ledeen (1984)
CDP-ethanolamine:1,2-diacylglycerol phosphoethanolamine transferase	Wu and Leeden (1980)
Choline kinase	Kunishita et al. (1987a)
CDP-choline:1,2-diacylglycerol phosphocholine transferase	Ledeen and Wu (1979)
Long-chain acyl-CoA synthase	Vaswani and Ledeen (1987)
Acyl-CoA:lysophospholipid acyltransferase	Vaswani and Ledeen (1989a)
ATP:1,2-diglycerol 3-phosphotransferase (diglyceride kinase)	Kahn and Moorell (1988)
Phospholipid catabolism	
Phosphatidate phosphatase (phosphatidic acid phosphatase)	Vaswani and Ledeen (1989b)
Phospholipase C toward phosphatidylcholine	Kanfer and McCartney (1989a, 1989b)
Phospholipase D toward phosphotidylcholine	Ledeen and Golly (1991)
Phosphoinositide metabolism	
ATP:phosphatidylinositol 4-phosphotransferase (PI kinase)	Deshmukh et al. (1978, 1981); Saltiel et al. (1987)
ATP:phosphatidylinositol-4-phosphate 5-phosphotransferase (PIP kinase)	Deshmukh et al. (1978, 1981)
Polyphosphoinositide phosphomonoesterases (PIP and PIP_2 phosphomonoesterases)	Deshmukh et al. (1982); Larocca et al. (1988); Palmer (1990)
Phospholipase C toward polyphosphoinositides	Deshmukh et al. (1982); Palmer (1990)
Membrane Transport	
Carbonic anhydrase	Cammer et al. (1976, 1977); Yandrasitz et al. (1976); Sapirstein and Lees (1978); Sapirstein et al. (1978)
Na^+,K^+-ATPase	Reiss et al. (1981); Zimmerman and Cammer (1982); Mrsuja et al. (1985)
5′-Nucleotidase	Kreutzberg et al. (1978); Cammer et al. (1980); Cammer and Zimmerman (1981); Heymann et al. (1984); Casado et al. (1988a, 1988b)
Protein metabolism	
Calcium-activated neutral protease (CANP or calpain)	Sato et al. (1982); Banik et al. (1985, 1991); Bertlet (1987); Yanagisawa et al. (1988); Chakrabarti et al. (1988, 1990a, 1990b)
Leucine aminopeptidase	Banik and Davidson (1969)
Acid protease	Bertlet et al. (1988)
Metalloproteinase	Chantry et al. (1989)
Protein kinase A	Johnson et al. (1971); Carnegie et al. (1973); Miyamoto and Kakiuchi (1974); Miyamoto (1975); Wu and Ahmad (1984); Bradbury et al. (1984)

(*Table continued on next page*)

TABLE 37–2. *Continued*

Enzyme	Reference
Protein kinase C	Turner et al. (1982, 1984); Kim et al. (1986); Murray and Steck (1986); Agrawal et al. (1990)
CA^{2+}-dependent, camodulin-stimulated kinase	Sulakhe et al. (1980a, 1980b); Endo and Hidaka (1980)
Myelin basic protein kinase	Yang (1986)
Ganglioside-stimulated kinase	Chan (1987)
Phosphorylated MBP phosphatase	Miyamoto and Kakiuchi (1974); MacNamara and Appel (1977); Wu et al. (1980); Yang et al. (1987)
Myelin basic protein (arginine) methyltransferase	Amur et al. (1986)
PLP fatty acyltransferase	Bizzozero et al. (1984); Konat et al. (1986); Ross and Braun (1988)
Acyl-proteolipid protein fatty esterase	Bizzozero et al. (1991, 1992)
Signal transduction-related	
Adenylate cyclase	Larocca et al. (1987a)
Phospholipases C and D	Kanfer and McCartney (1989a, 1989b); Ledeen and Golly (1991)
Others	
Esterase (nonspecific)	Rumsby et al. (1973); Tada et al. (1986)
Glutathione-*S*-transferase	Tansey and Cammer (1991)

activities: (1) lysosomal enzyme with lower optimal pH, (2) microsomal enzyme with optimal pH of 6.0, and (3) myelin-associated enzyme with an optimal activity at pH 7.2. The myelin enzyme was discriminated from the microsomal enzyme by their detergent requirement. The developmental profile of the myelin enzyme correlated with the accumulation of myelin (Eto and Suzuki, 1973b). Differing from most myelin-associated enzymes, cholesterol ester hydrolase activity in spinal cord myelin increases much more rapidly during early development and reaches the adult level three times higher as compared with that in brain myelin (Eto and Suzuki, 1973b). About 60 to 70% of the enzyme activity at pH 7.2 in whole brain was recovered in the myelin preparation with a 10-fold increase in relative specific enzyme activity. These results strongly suggest that cholesterol ester hydrolase is a specific marker enzyme for myelin. On the other hand, a recent study with rat, mouse, and human brains demonstrated that the enzyme activities in myelin and microsomes could not be distinguished from each other in respect to pH optimum and detergent and phospholipid requirement (Johnson and Shah, 1986). Thus, the specificity of the myelin enzyme remains to be determined.

UDP-Galactose:Ceramide Galactosyltransferase

UDP-galactose:ceramide galactosyltransferase is an enzyme that catalyzes the transfer of galactose to ceramide to synthesize galactosyl ceramide, one of the major lipid components in myelin. This enzyme activity was detected in myelin as well as microsomal fractions with similar specific activities (Nescovic et al., 1973; Constantino-Ceccarini and Suzuki, 1975). The presence of an intrinsic galactosyltransferase activity in myelin has been demonstrated by fractionation (Koul et al., 1980; Lees and Sapirstein, 1983) and immunocytochemical studies (Roussel et al., 1987). While both myelin and microsomal enzymes exhibited similar enzymatic properties, their developmental patterns differ from each other; the activity of myelin enzyme increases with active myelination and decreases thereafter to the adult level (Brenkert and Radin, 1972; Constantino-Ceccarini and Morell, 1972; Koul et al., 1980). The developmental profiles of the enzyme activity in different central nervous system regions appear to correspond with the timing of myelin deposition in these regions (Koul and Jungawala, 1986; Monge et al., 1988). Recently, it has been demonstrated that the enzyme activities directed toward HFA- and NFA-ceramides have different intracellular and myelin distribution in rat brain tissues, implying that these two activities may be catalyzed by different enzyme entities (Sato et al., 1988).

Neuraminidase (Sialidase)

Neuraminidase catalyzes the cleavage of terminal sialic acid residues from glycoconjugates such as sialoglycoproteins and gangliosides and plays a key role in the catabolism of these sialoglycoconjugates. The existence of an intrinsic neuraminidase activity in brain myelin was first suggested by Yohe et al. (1983) and, thereafter, characterized using a ganglio-

side (GM3) and nonganglioside substrates (*N*-acetylneuramin-lactitol) (Saito and Yu, 1986; Yohe et al., 1986). The enzyme activities directed toward these two substrates have been assumed to be catalyzed by a single enzyme (Saito and Yu, 1986). It is interesting that the enzyme can slowly hydrolyze GM1, which is usually resistant to most viral and bacterial neuraminidases. This finding is consistent with observations such as the slow turnover of GM1 in myelin (Suzuki, 1970; Ando et al., 1984) and the existence of asialo-GM1 in myelin (Kusunoki et al., 1986). The enzyme activities in myelin increase during active myelination, followed by a sharp decline to the adult level (Saito and Yu, 1992). Differing from central nervous system myelin, the neuraminidase is hardly detected in sciatic nerve myelin (Yohe et al., 1986). A study on the hydrolysis of membrane gangliosides by endogenous neuraminidase in myelin provided evidence that myelin-associated neuraminidase may play a critical role in the developmental changes in myelin gangliosides (Saito and Yu, 1992). It has also been suggested that the enzyme may function as a membrane adhesion molecule because a specific interaction exists between the enzyme and GM1 and there is a specific adhesion of oligodendroglial cells to GM1 (Saito and Yu, 1993). Thus, the myelin-associated neuraminidase not only plays a critical role in ganglioside metabolism in myelin, but also may be involved in the formation and maintenance of the myelin structure.

Phospholipid-Synthesizing Enzymes

Myelin contains a variety of enzymes involved in the synthesis and degradation of phospholipids. Regarding phospholipid-synthesizing enzymes, myelin has been shown to contain a set of enzyme activities that are required for the synthesis of phosphatidylethanolamine (PE). The first enzyme to be discovered was CDP-choline:1,2-diacylglycerol phosphoethanolamine transferase, which catalyzes the final step of PE synthesis (Wu and Ledeen, 1980). Additional enzymes required for PE synthesis are ethanolamine kinase and CTP:phosphoethanolaminecytidyltransferase, and both have been shown to be associated with the myelin membrane (Kunishita and Ledeen, 1984; Kunishita et al., 1987a). These findings suggest that PE can be synthesized *in situ* in central nervous system myelin. Myelin is also capable of carrying out the biosynthesis of phosphatidylcholine, as suggested by the existence of enzyme activities such as choline kinase (Kunishita et al., 1987b) and CDP-choline:1,2-diacylglycerol phosphocholine transferase (Ledeen and Wu, 1979).

Long-chain acyl-CoA synthetase is required for acylation in phospholipid synthesis and has been found in myelin (Vaswani and Ledeen, 1987). This enzyme can utilize oleic and arachidonic acids as the substrates. Acyl-CoA:lysophospholipid acyltransferase in myelin catalyzes the transfer of acyl chains to various type of lysophosphatidylphospholipids, especially lysophosphatidylcholine (Vaswani and Ledeen, 1989a). Regarding the metabolism of diglyceride, a metabolic study with ^{32}P-phosphate and ^{3}H-glycerol suggested the existence of ATP:1,2-diacylglycerol 3-phosphotransferase (diglyceride kinase) activity in myelin (Kahn and Morell, 1988). Evidence has been presented that the radiolabeled phosphatidic acid in myelin, an important metabolite in the phospholipid signal transduction pathway, may be generated mainly by phosphorylation of diglyceride, which is supplied through the breakdown of polyphosphoinositides by phospholipase C. This hypothesis is supported by the finding that addition of acetylcholine stimulates the formation of phosphatidic acid (Kahn and Morell, 1988).

Phospholipid-Catabolizing Enzymes

Phosphatidic acid phosphatase plays a key role in phospholipid metabolism in that it provides diacylglycerol (diglyceride), which is an essential intermediate in the synthesis of phospholipids. The activity of this enzyme has been found in rat brain myelin (Vaswani and Ledeen, 1989b). Myelin also contains a phospholipase C activity that hydrolyzes phosphatidylcholine, generating diacylglycerol and phosphorylcholine. The specific activity of this enzyme was about threefold higher than that in brain homogenate (Kanfer and McCartney, 1989a, 1989b). The existence of phospholipase D activity directed toward phosphatidylcholine has also been reported recently (Ledeen and Golly, 1991).

Phosphoinositide-Metabolizing Enzymes

Myelin contains a relatively high level of polyphosphoinositides (Hauser et al., 1971; Eichberg and Hauser, 1973). The distribution of phosphoinositides in the myelin sheath may be different for the various phosphoinositide species; phosphatidylinositol is distributed evenly in three myelin subfractions, while phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2) are concentrated in the light myelin fraction (Deshmukh et al., 1980). Metabolic studies with ^{32}P-phosphate have indicated that the phosphate groups of phosphoinositides in myelin undergo rapid turnover

(Deshmukh et al., 1981; Kahn and Morell, 1988). These findings suggest the existence of synthetic and degradative activities for phosphoinositides in myelin. Phosphatidylinositol and phosphatidylinositol-4-phosphate (PIP) kinase activities, which catalyze the formation of PIP and PIP_2, respectively, have been detected in purified myelin (Schacht, 1976); both kinase activities are highest in the heavy myelin fraction although appreciable levels are also present in other subfractions (Deshmukh et al., 1978). Phosphatidylinositol kinase has recently been purified from bovine brain (Saltiel et al., 1987).

As for enzyme activities responsible for the degradation of phosphoinositides, relatively high levels of PIP and PIP_2 phosphomonoesterase activities have been detected in myelin (Deshmukh et al., 1982). The activities directed toward PIP and PIP_2 show different developmental profiles, suggesting that they may be ascribed to distinct enzyme entities (Palmer, 1990). Myelin contains both activities of phospholipase C directed toward PIP and PIP_2, generating inositol 1,4-phosphate (IP_2) and inositol 1,4,5-triphosphate (IP_3), respectively (Keough and Thompson, 1970; Deshmukh et al., 1982). These activities may play an important role in signal transduction in myelin.

Transport-Related Enzymes

It has been suggested that ion transport is an important process in the myelin membrane and may be involved in many physiological functions of the membrane. The myelin membrane has been shown to possess ion transport mechanisms (de Cozar et al., 1987; Robertson et al., 1989) including K^+-channels (Morell et al., 1990). Of several components of myelin, proteolipid proteins may function as ionophores and may be involved in the ion transport processes (Ting-Beall et al., 1979; Helynck et al., 1983). Lees and Sapirstein (1983) have proposed that carbonic anhydrase and Na^+,K^+-ATPase may play an important role in controlling K^+ levels at the node of Ranvier. It has also been demonstrated that these two enzymes are involved in transmembrane movement of fluid in many cells. This may be the case for myelin, as suggested by a recent study demonstrating that acetazolamide, an inhibitor of carbonic anhydrase, improves edema-induced vacuolar degeneration of brain myelin induced by triethytin (Yanagisawa et al., 1990).

Carbonic anhydrase is an enzyme that catalyzes formation of carbonic acid (H_2CO_3) from CO_2 and H_2O. Since neurons are practically devoid of this enzyme activity, the myelin enzyme may play a role in the removal of CO_2 that is produced in excess during elevated neuronal activity. The generated carbonic acid is dissociated into bicarbonate (HCO_3^-) and proton (H^+) at physiological pH, forming the proton gradient across the membrane (Giacobini, 1987). In brain tissues, carbonic anhydrase exists in both soluble and membrane-bound forms (Sapirstein et al., 1978). The enzyme is located in myelin (Cammer et al., 1976, 1977; Sapirstein and Lees, 1978) in addition to astroglial and oligodendroglial cells (Ghandour et al., 1979; Kumpulainen and Korhonen, 1982; Cammer and Tansey, 1988a, 1988b). Among myelin subfractions, the medium and heavy myelin are more enriched with the enzyme activity (Sapirstein et al., 1978). Myelin-associated carbonic anhydrase having an apparent molecular weight of 30 kD has enzymatic and immunological properties similar to the type C isoenzyme isolated from erythrocytes.

Na^+,K^+-ATPase plays a crucial role in the Na^+-K^+ pump mechanism. While previous studies have detected a Na^+,K^+-ATPase activity in isolated myelin, this activity has been attributed to contaminating membranes mainly because of the low recovery of the activity in myelin and relatively low specific activity (Norton and Poduslo, 1973; DeVries et al., 1978; Danks and Matthieu, 1979). Thereafter, evidence has been accumulated suggesting that this enzyme activity is intrinsic to myelin (Zimmermann and Cammer, 1982). Na^+,K^+-ATPase activity in myelin could be distinguished from that in microsomes in respect to effects of detergent, discontinuous temperature for activity, and affinity to strophantidin (Reiss et al., 1981). A discrepancy between developmental profiles of the enzyme activities in myelin and brain homogenates also supports the existence of the enzyme activity intrinsic to myelin (Zimmermann and Cammer, 1982). These results are strongly supported by an immunocytochemical study demonstrating the presence of K^+-dependent *p*-nitrophenylphosphatase (K^+-NPPase) activity, a component of the Na^+,K^+-ATPase complex, in myelin (Mrsulja et al., 1985). The enrichment of K^+-NPPase activity in the paranodal region as compared to compact myelin supports the hypothesis that this region may be the main site for ion transport (Mackenzie et al., 1984).

5′-Nucleotidase hydrolyzes 5′-AMP to produce adenosine, which functions as a neuromodulator through its effects on Ca^{2+}-transport and adenyl cyclase activity (McCarthy and DeVellis, 1980; Phillis and Wu, 1981). While this enzyme is widely distributed to plasma membranes of different cell types, it is also an integral enzyme of the myelin membrane (Cammer et al., 1980). The highest specific activity

of 5′-nucleotidase is found in heavy myelin subfractions (Cammer and Zimmermann, 1981; Casado et al., 1988a, 1988b). Immunocytochemical studies have demonstrated the presence of the enzyme at the surface of the myelin sheath, between the axolemmae and innermost myelin lamellae, and within the myelin lamellae as well as glial plasma membranes (Kreutzberg et al., 1978; Heymann et al., 1984; Casado et al., 1988a, 1988b). The activity of 5′-nucleotidase in rat brain myelin increases almost fourfold during the developmental period from 20 to 90 days of age, while the activities of CNPase and carbonic anhydrase are relatively constant (Cammer and Zimmermann, 1981). Consequently, the recovery of 5′-nucleotidase in myelin (21%) resembles that of cyclic nucleotide phosphodiesterase (28%), suggesting that myelin may be the predominant, although not exclusive, locus of 5′-nucleotidase in adult brain.

The π form of glutathione-*S*-transferase (GST) has recently been demonstrated to be intrinsic to central nervous system myelin, as well as to oligodendrocytes (Tansey and Cammer, 1991). The GSTs function in intracellular transport of hormones and removal of toxins from cells (Tansey and Cammer, 1991), and may thus represent a protective mechanism for the myelin sheath.

Calcium-Activated Neutral Protease and Other Proteases

The presence of protease activities in central nervous system myelin was initially suggested some time ago (Marks et al., 1976; Smith, 1977). Of the protease activities, calcium-activated neutral protease (CANP, calpain) has drawn much attention because of its possible involvement in demyelinating diseases (Banik et al., 1992). The existence of intrinsic CANP activity has been demonstrated by autodigestion of myelin proteins during incubation of myelin membranes in the presence of Ca^{2+}. Both MBP and MAG appear to be good substrates for the enzyme (Sato and Miyatake, 1982; Sato et al., 1982; Banik et al., 1985). While PLP or Wolfgram proteins are fairly resistant to the protease action, the addition of Triton X-100 and EGTA (no Ca^{2+}) causes preferential degradation of PLP relative to MBP implying the involvement of a noncalcium-dependent protease(s) for the degradation of PLP (Banik et al., 1985). More recently, the presence of CANP in purified myelin has been confirmed using an anti-chicken CANP antibody (Yanagisawa et al., 1988). Partially purified CANP from myelin is activated by Ca^{2+} in the millimolar range (mCANP or calpain II). Since the intracellular Ca^{2+} concentration is much lower than this level, this enzyme may be autolyzed into a more sensitive μM form (μCANP), which is more active *in vivo*. A recent observation that myelin mCANP can be activated by gangliosides may also be of some interest for considering the regulatory mechanism of the enzyme activity (Chakrabarti et al., 1990a, 1990b).

In addition to CANP, aminopeptidase (Banik and Davison, 1969), acid protease (Berlet et al., 1988), and metalloproteinase (Chantry et al., 1989) have been shown to be associated with myelin. The activity of acid protease is assumed to be due to cathepsin D-like endopeptidase, whereas the metalloprotease cleaves many bonds in MBP, producing only trichloroacetic acid-precipitable peptides.

Protein Kinases and Phosphatase-Related Enzymes

Phosphorylation and dephosphorylation are the major reactions in protein metabolism in myelin. Myelin proteins, especially MBPs, have been shown to be radiolabeled rapidly after injection of ^{32}P-phosphate into rats (Carnegie et al., 1974; Miyamoto and Kakiuchi, 1974; Miyamoto et al., 1974; Kim et al., 1986). The phosphate groups of MBPs are metabolized with half-lives of minutes, contrasting to their polypeptide backbones, which have turnover rates on the order of months (Fisher and Morell, 1974; McNamara and Appel, 1977; Shapira et al., 1981; DesJardins and Morell, 1983). The phosphorylation reaction has been observed even in regions of compact myelin (DesJardins and Morell, 1983). These findings suggest the presence of protein kinase and phosphatase activities in myelin.

To date, different types of protein kinase activities have been found in myelin, including cAMP-dependent kinase (protein kinase A) (Carnegie et al., 1973; Miyamoto, 1975; Wu and Ahmad; 1984), Ca^{2+}-dependent, calmodulin-stimulated kinase (Sulakhe et al., 1980a, 1980b; Endo and Hidaka, 1980), and Ca^{2+}-dependent, phospholipid-stimulated kinase (protein kinase C) (Turner et al., 1982, 1984; Kim et al., 1986; Murray and Steck, 1986). Activity of protein kinase A appears to be associated loosely with myelin membranes and shows properties similar to those seen in microsomes (Miyamoto et al., 1978; Wu and Ahmad, 1984). A MBP-specific kinase has been shown to function as an activator for ATP,Mg^{2+}-dependent protein phosphatase (Yang, 1986). Ganglioside-stimulated kinase does not phosphorylate MBP preferentially (Chan, 1987).

Intrinsic activity of protein phosphatase in myelin has also been shown (Miyamoto and Kakiuchi, 1975; MacNamara and Appel, 1977). An enzyme

with an apparent molecular weight of 46 kD has been purified and its substrate specificity characterized (Wu et al., 1980). A recent study suggests that an inactive/latent phosphatase with a molecular weight of 350 kD (designated LP-2) is the most predominant protein phosphatase responsible for the dephosphorylation of brain myelin (Yang et al., 1987).

The physiological significance of these enzyme activities in myelin has been strengthened by the finding that myelin contains various types of control systems for the activities. Myelin possesses an intrinsic adenylate cyclase activity that is responsible for the production of cyclic AMP, an activator for protein kinase A (Larocca et al., 1987a).

Receptors, Second-Messenger Systems, and Mitogenic Activity

The traditional view of myelin as an insulating membrane has been forced to undergo expansion and revision by reports in recent years of myelin-intrinsic signal transduction systems. A number of substrates and enzymes known to be linked to such systems have been known to exist in myelin for some time. For example, phosphoinositides had been observed in isolated myelin prior to their implication in the signal transduction pathway involving phospholipase C hydrolysis to produce second messengers (Kahn and Morell, 1988). Studies from several laboratories intimate that muscarinic receptors are intrinsic to myelin and that these receptors are linked to formation of second messengers (reviewed by Ledeen, 1992). Binding of ligand to these receptors can activate adenylate cyclase with subsequent activation of protein kinase A; alternatively, receptor stimulation may lead to phospholipase C hydrolysis of phosphoinositides to produce diacylglycerol, which leads to stimulation of protein kinase C, and inositol 1,4,5-triphosphate, which acts to mobilize calcium (Larocca et al., 1987b; Kahn and Morell, 1988; Eichberg et al., 1989). The GTP-binding family of proteins seems an obvious candidate for a common denominator in all of these pathways. In fact, several laboratories have now reported the presence of a number of G proteins associated with myelin (Braun et al., 1990b; Larocca et al., 1991), thus providing a mechanism for transmitting signals from receptor to intracellular effector molecules. The *in vivo* source of extracellular acetylcholine or other cholinergic agonist has yet to be resolved.

A series of studies have demonstrated that a myelin-enriched fraction isolated from either the peripheral or central nervous system contains an intrinsic mitogenic activity (reviewed by Baichwal and De Vries, 1992). This mitogenic activity is directed toward Schwann cells and mediated by phagocytosis of the myelin membrane by macrophages. Evidence has been presented that the mitogen itself is a polypeptide related to MBP (Baichwal and De Vries, 1989). The mitogen characterized in these studies is postulated to be responsible for the Schwann cell proliferation associated with Wallerian degeneration *in vivo* (Baichwal and De Vries, 1992).

CONCLUDING REMARKS

Our understanding of myelin biochemistry has been greatly facilitated by the development by William Norton and his collaborators in the 1970s of a convenient procedure for isolating highly purified myelin preparations. Most of the early studies were directed toward defining the chemical composition of myelin. These early studies reinforced the long-held notion that the major function of myelin is to facilitate nerve conduction. More recent research has given rise to the idea that myelin is not only an inert insulator, but is actually metabolically active with many myelin-associated enzymes and voltage-dependent ion transport systems. With the advent of contemporary molecular biological techniques, great strides have been made in determining the structure and function of myelin genes as well as in delineating mutations involved in a variety of genetic disorders affecting myelination and the maintenance of myelin structure. Despite the recent triumphs of molecular genetics and molecular biology, the regulatory mechanisms for myelin synthesis and assembly remain obscure. Moreover, the biological and physiological functions of many myelin proteins and lipids are still not fully understood. The future of myelin research undoubtedly lies in addressing such problems.

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38 Molecular biology of myelination

ANTHONY T. CAMPAGNONI

Myelination is a developmentally regulated event that begins postnatally in the rat and mouse brain and within the third fetal trimester in the human spinal cord. The expression of the myelin protein genes and the synthesis and assembly of these proteins into myelin represent one of the most intriguing aspects of modern molecular neurobiology. The genes encoding a number of myelin proteins have now been isolated and their structures have been determined. Table 38-1 lists myelin proteins for which the genes have been cloned and some of the characteristics of these proteins. Table 38-2 lists characteristics of the myelin protein genes that have been examined to date.

The synthesis and assembly of myelin proteins into the membrane is a complex event involving a number of steps. Research over the past decade indicates that regulation of the expression of these genes occurs at a number of different levels, including (1) promoter choice, (2) transcription, (3) splicing, (4) mRNA stability, (5) translation, and (6) posttranslational processing. This chapter briefly examines the molecular biology of myelination from the perspective of the myelin protein genes, their expression into mRNA and protein, and the assembly of their protein products into myelin. Since a number of reviews have appeared since 1988 (Campagnoni, 1988; Campagnoni and Macklin, 1988; Lemke, 1988; Nave and Milner, 1989; Ikenaka et al., 1991; Mikoshiba et al., 1991), this chapter emphasizes the most recent findings in this area. The reader is encouraged to consult these reviews for information on earlier studies.

From a historical perspective, the myelin basic protein and proteolipid protein were among the first nervous system proteins to be isolated and characterized biochemically because of their relatively high abundance in nervous tissue. It is not surprising, then, that they also were among the first cDNAs to be isolated from brain cDNA libraries. In the case of myelin basic protein (MBP) cDNAs, degenerate DNA probes, based on the known protein sequence, were used to isolate cDNAs from brain libraries (Roach et al., 1983; Zeller et al., 1984). One of the first proteolipid protein (PLP) cDNAs to be isolated occurred as part of a screen for "brain-specific" clones (Milner et al., 1985) and its high abundance in adult rat brain resulted in its isolation.

Figures 38-1, 38-3, 38-4, and 38-5 diagram the structures of the myelin protein genes along with the RNA splice products that have been identified to date. The genes of many of the myelin proteins located in the central nervous system have been found to be alternatively spliced to produce at least two polypeptide isoforms. For several of the myelin proteins [MBP, PLP, 2′,3′cyclic nucleotide 3′phosphodiesterase (CNPase), and myelin-associated glycoprotein (MAG)] the proportions of the individual isoforms expressed changes with development, thus the splicing patterns of the genes are developmentally regulated.

Golli-mbp GENE

Structure

The structure of the *MBP* gene was the first of the myelin protein genes to be elucidated. The "classic" *MBP* gene consists of seven exons distributed over a 32 kb stretch of chromosome 18 in mouse (Roach et al., 1985). The gene maps to human chromosome 18 (Sparkes et al., 1987) and rat chromosome 1 (Koizumi et al., 1991). At least six transcripts are expressed from this gene in the mouse through alternative splicing of three exons. The alternatively spliced MBP mRNAs encode MBP isoforms that range in molecular mass between 14 and 21 kD. The splicing of the transcripts is developmentally regulated. For example, those containing exon 2 (i.e., encoding the 17 and 21.5 kD isoforms) appear early in myelination and decline with development.

Recently, the *MBP* gene has been found to be included in a larger transcription unit, called the *Golli-mbp* gene (Campagnoni et al., 1993; Pribyl et al., 1993). The exons and promoter region comprising the MBP gene are included within the *Golli-mbp* transcription unit, which is approximately 105 kb in length in the mouse (Figure 38-1). A 36 kb region of the *Golli-mbp* gene that contains the MBP-encod-

TABLE 38-1. *Myelin Proteins for Which cDNAs Have Been Cloned*

Protein	CNS/PNS	Mass (kDa)	Characteristics	References[a]
CNPase	CNS and PNS	44–48	No known function. Found in myelin and oligodendrocyte plasma membranes. Early marker of oligodendrocytes	Sprinkle, 1989; Tsukada and Kurihara, 1992
MAG	CNS and PNS	~100 (glycosylated) 67/72 (apoproteins)	Principal glycoprotein of the CNS. Believed to be an adhesion molecule involved in the interaction between the axon and the myelin sheath. Implicated in some peripheral neuropathies	Mendell et al., 1985; Quarles et al., 1992; Tatum, 1993
MBPs	CNS and PNS	14–21.5	Located at major dense line. Peripheral membrane protein. Encephalitogenic, autoimmunogenic. pI > 10.5	Campagnoni, 1988; Campagnoni and Macklin, 1988
MOG	CNS	26–28	Located at external surfaces of myelin sheath and oligodendrocytes. May be involved in autoimmune demyelinating events.	Bernard and Kerlero de Rosbo, 1991
OMgp	CNS	120	Glycosylated protein. Located in oligodendrocyte membranes and at paranodal region of myelin sheath.	Mikol and Stefansson, 1988; Mikol et al., 1990b
P_0 protein	PNS	28	Major glycoprotein of the PNS. Integral membrane protein, probably important in myelin compaction through homotypic interactions between lamellae.	D'Urso et al., 1990; Filbin et al., 1990; Uyemura et al., 1992
P_2 protein	PNS and CNS (minor)	~15	A basic protein that binds fatty acids and retinoids. An autoimmunogen that induces experimental allergic neuritis.	Martenson and Uyemura, 1992; Narayanan et al., 1988; Veerkamp et al., 1991
PMP-22/PAS II	PNS	18	Minor glycoprotein of PNS myelin.	Snipes et al., 1992; Spreyer et al., 1991
PLP/DM20	CNS and PNS	25 (DM20) 30 (PLP)	Very hydrophobic integral membrane protein. Highly conserved amino acid sequence. Encephalitogenic.	Lees and Bizzozero, 1992; Nave and Milner, 1989

CNS, central nervous system; PNS, peripheral nervous system; CNPase, 2′,3′-cyclic nucleotide-3′-phosphodiesterase; MAG, myelin-associated glycoprotein; MBPs, myelin basic proteins; MOG, myelin/oligodendrocyte glycoprotein; OMgp, oligodendrocyte-myelin glycoprotein; PMP-22/PAS II, peripheral nervous system myelin protein; PLP/DM20, proteolipid proteins.
[a]Primarily reviews.

ing exons 5B-11 can function as an independent transcription unit. It can be introduced into transgenic mice and expressed within oligodendrocytes of the central nervous system with appropriate developmental specificity. It also can be introduced into *shi* mice (which have a deletion of exons 7–11), with a subsequent reduction in the clinical signs of the neurological disorder caused by the mutation (Readhead et al., 1987). Sequences up to 4 kb upstream of *Golli-mbp* exon 5B can be used to drive the expression of oncogenes (Hayes et al., 1992), MHC (major histocompatibility complex) molecules (Turnley et al., 1991), and reporter genes (Gow et al., 1992; Miskimins et al., 1992) in transgenic mice. Exon 5A of the *Golli-mbp* gene also can drive the expression of reporter genes in transfected cells (Miura et al., 1989; Devine-Beach et al., 1990). Thus, the *MBP* gene is part of a rather complex genetic locus, and is an example of a "gene within a gene." In this respect, the *Golli-mbp* gene is similar to the neurofibromatosis gene in that they are both large transcription units that encompass smaller genes (Viskochil et al., 1991). However, they are distinctly different in that the smaller MBP portion of

TABLE 38-2. *Some Characteristics of Myelin Protein Genes*

Gene	Number of Exons	Chromosome	Length (kb)	Comments	References
CNPase	4	3,11 (m) 17 (h)	~6 (m) ~9 (h)	Relatively small gene with two transcription start sites and alternative splicing pattern.	Douglas et al., 1992; Kurihara et al., 1990
Golli-mbp (*MBP*)	11	18 (h, m) 1 (r)	~105 (m) ~179 (h)	Very complex genetic locus. *MBP* gene entirely encompassed within larger transcription unit. Alternatively spliced.	Campagnoni et al., 1993; Kamholz and Wrabetz, 1992; Pribyl et al., 1993
MAG	13	7 (m) 19 (h)	~16 (r)	Member of the immunoglobulin gene superfamily. Alternatively spliced.	Milner et al., 1990
OMgp	2	17 (h)	~3	Entire gene contained within an intron of the neurofibromatosis gene.	Mikol et al., 1990; Viskochil et al., 1991
P_0	6	1 (m)	~7 (m, r)	Expression restricted to Schwann cells. Ig-related domains split between two exons.	Lemke et al., 1988; Uyemura et al., 1992
P_2	4	?	~4.5	Member of the fatty acid-binding protein family of genes. Highly conserved through evolution.	Narayan et al., 1991; Medzihradszky et al., 1992
PLP/DM20	7	X (h, m)	~17	Gene structure strongly conserved throughout evolution. Alternatively spliced.	Macklin, 1992; Hudson and Nadon, 1992; Nave and Milner, 1989

h, human; m, mouse; r, rat. For gene abbreviations see Table 38.1.

the *Golli-mbp* gene is not simply included within an intron of the larger transcription unit, but shares alternatively spliced exons in common.

The splicing pattern of *Golli-mbp* is very complex, both with respect to the numbers of products expressed and their developmental regulation. In the mouse, the gene contains three transcription start sites (at exons 1, 4, and 5B in Figure 38-1) each of which becomes active at different times during pre- and postnatal brain development. Transcription start site 1 expresses several *Golli* RNA splice products that contain exons 1, 2, and 3 of the gene spliced

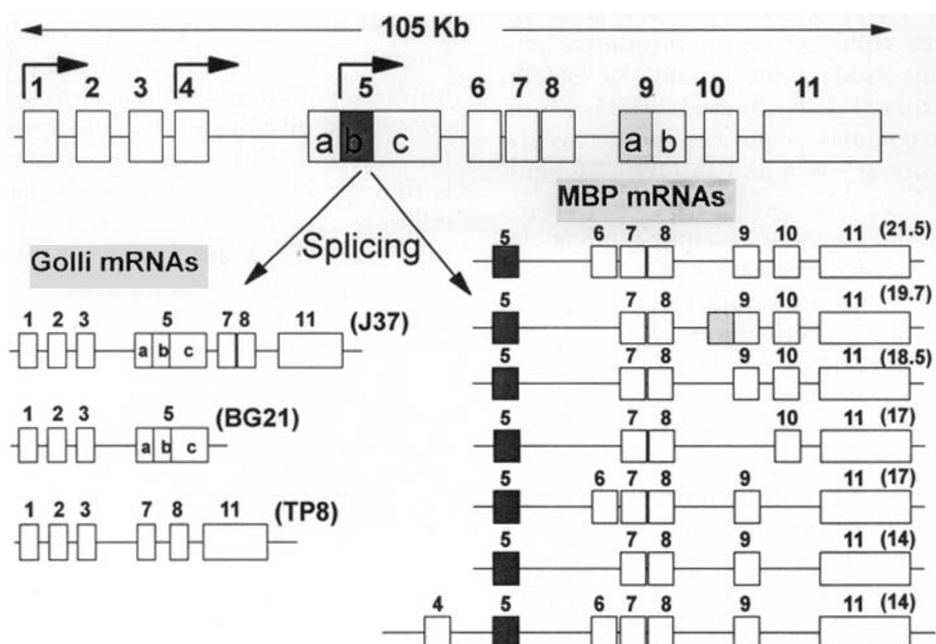


FIG. 38-1. Illustration of the structure and splicing pattern of the *Golli-mbp* gene. The names of the Golli mRNA products (*in parentheses*) are taken after those published by Campagnoni et al., 1993. The individual MBP mRNAs are identified (*in parentheses*) by the MBP isoform they encode. The three *arrows* above the gene indicate the three transcription start sites of the gene.

into MBP-encoding exons (see Figure 38-1). The second start site at exon 4 (Kitamura et al., 1990) and the third transcription start site at exon 5B express MBP mRNAs. The expression of at least six alternatively spliced forms of MBP mRNAs generated from the major (third) transcription start site has been well documented (see Aruga et al., 1991).

Recently, the human gene has been cloned and its organization is similar to that of the mouse gene except that it is much larger, approximately 179 kb (Pribyl et al., 1993). It also expresses alternatively spliced products, some of which are similar but not identical in structure to the mouse products.

Transcriptional Regulation

Identification of the *cis* genetic elements and the *trans*-acting factors regulating the tissue, cell, and developmental specificity of the *MBP* gene has drawn the attention of several laboratories in recent years. It has generally been felt that the regulation of the gene is complex, involving multiple sites within the region upstream of the MBP transcription start site and encompassing exon 5A of the *Golli-mbp* gene, where *trans*-acting factors that bind to different parts of the promoter interact to regulate the activity of the MBP transcription unit (Mikoshiba et al., 1991). Accordingly, this region has been isolated, analyzed, and used to define putative regulatory elements. The MBP promoter does not contain conventional TATA or CAAT boxes, that is, regulatory sequences common to all promoters and believed to be involved in the binding of general transcription factors (Takahashi et al., 1985; Miura et al., 1989). It contains sequences similar to the SV40 enhancer core at −648 and an NFI/CTF binding site at −125.

Recently, a thyroid hormone receptor binding element has been identified between −163 and −186 nt upstream of the transcription start site (Farsetti et al., 1991). Mikoshiba and coworkers (Mikoshiba et al., 1991) and Khalili and coworkers (Devine-Beach et al., 1990, 1992) have defined two regions of the promoter they call the "proximal" (i.e., +60 to ~ −50) and "distal" (i.e., ~ −50 to ~ −300) regions. Within each of these regions a number of sequences have been reported to confer either tissue or cellular specificity to the expression of the gene (Tamura et al., 1989, 1991; Devine-Beach et al., 1990, 1992). The focus of most of these studies has been on a region 1.3 kb upstream of the *MBP* gene, primarily within the +60 to −300 nt region of the gene and its upstream region. In another approach to defining elements important in the tissue-specific regulation of *MBP* gene expression, Gow et al. (1992) have recently reported that, in transgenic mice, the 1.3 kb upstream region of the *MBP* gene does not contain the information necessary to permit expression of the gene in the peripheral nervous system.

Posttranscriptional Regulation

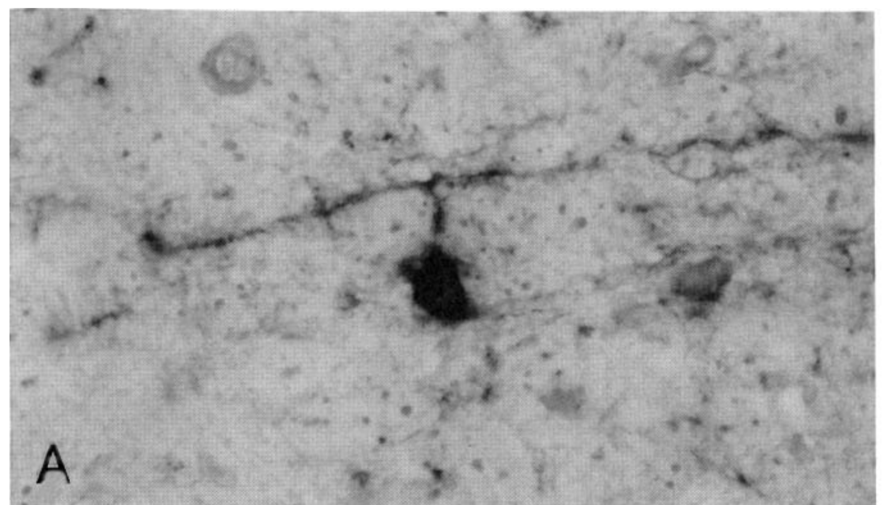
There is growing evidence that expression of the MBP transcription unit may be regulated at the posttranscriptional level. Several groups have reported posttranscriptional regulation by steroids or thyroid hormone by mechanisms including MBP mRNA stabilization or direct effects on translation rates (Kumar et al., 1989; Verdi and Campagnoni, 1990; Cabacungan et al., 1991).

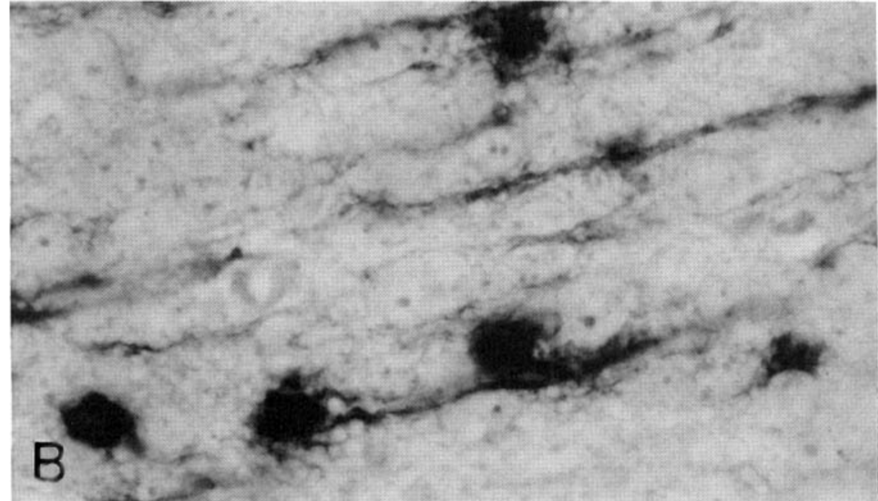
One of the most interesting features of posttranscriptional events of *MBP* gene expression is the well-documented phenomenon of MBP mRNA translocation. The MBPs are highly cationic polypeptides that can interact with virtually any negatively charged molecule. Therefore, a mechanism is required for targeting MBPs to the oligodendrocyte processes without inappropriate interactions of MBP polypeptides with other cellular components. This appears to be accomplished through the translocation of MBP mRNAs from the cell somas to the oligodendrocyte processes and channels that infiltrate the myelin sheath. This has been observed in many studies both *in vivo* (Trapp et al., 1987; Verity and Campagnoni, 1988) and *in vitro* (Amur-Umarjee et al., 1990b). Figure 38-2 shows an *in situ* hybridization result, which clearly localizes the MBP mRNAs within oligodendrocyte cell bodies, processes, and myelin sheaths. The myelin sheaths stain for MBP mRNA because the messages are localized within the cytoplasmic channels that surround and infiltrate the sheath. The extensive dispersion of MBP mRNAs within these cytoplasmic channels, recognized by the probes, provides an *in situ* hybridization result that looks very much like results obtained by injection of oligodendrocytes with horseradish peroxidase (Ransom et al., 1991). Very recently, Ainger et al. (1993) have demonstrated the existence of two sites within the 3′UT region of the MBP mRNA that are involved in targeting these messages to oligodendrocyte somas or processes.

MYELIN PROTEOLIPID PROTEIN (*PLP/DM20*) GENE

Structure and Localization

The myelin proteolipid proteins, PLP and DM-20, constitute about 50% of the protein in the myelin





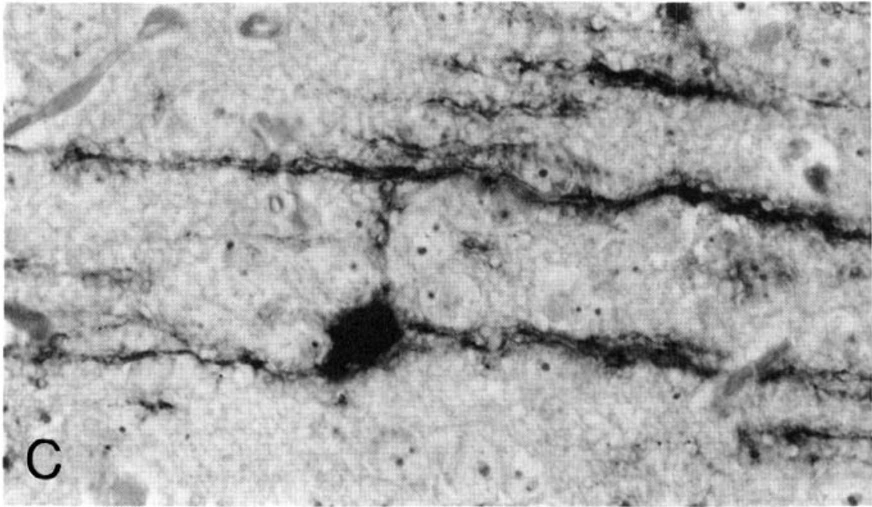


FIG. 38-2. A montage of three areas of mouse midbrain showing the *in situ* hybridization histochemical localization of MBP mRNA using digoxigenin-labeled riboprobes. (A) MBP mRNA within a single oligodendrocyte extending a process to a myelinated fiber that also contains MBP mRNA. (B and C) Several regions of myelinated fibers that contain MBP mRNA, presumably evident because of their localization within the cytoplasmic channels coursing through the sheath. In addition, they show several oligodendrocyte cell bodies also filled with MBP mRNA. In some cases the bodies are connected to the myelinated fibers through MBP mRNA-containing processes. In Figure C the oligodendrocyte appears to possess MBP mRNA-containing processes connected to at least two MBP mRNA-containing myelinated fibers. [Photomicrographs courtesy of Dr. Charles F. Landry.]

sheath and, as such, are among the most abundant proteins in the central nervous system. The amino acid sequences of the PLPs have been deduced from their corresponding cDNAs in many species, and their strong conservation among species has been noted by many investigators (for reviews see Nave and Milner, 1989; Hudson and Nadon, 1992; Macklin, 1992).

The *PLP/DM20* gene, located on the X chromosome in mouse, rat, and man, is ~17 kb in length

and consists of 7 exons (Figure 38-3). The myelin PLP was thought to be the only proteolipid in myelin until the early 1970s when a second proteolipid, called DM20, was identified and characterized by Agrawal et al. (1972). On the basis of peptide mapping studies of the purified polypeptides, Trifilieff et al. (1985) proposed that DM20 differed from PLP by an internal deletion of ~40 amino acids. Molecular biological studies subsequently established the location of a 35-amino acid deletion in the protein (Nave et al., 1987). This region is encoded in exon 3B of the gene.

The *PLP/DM20* gene is alternatively spliced to produce two mRNA products that encode the PLP and DM20 polypeptides. The gene also possesses three polyadenylation signals within the last exon encoding the 3′ untranslated region of the mRNA. The utilization of any or all of these sites generates a family of PLP/DM20 mRNAs of ~1.6, ~2.4, and ~3.2 kb. The proportions of these size classes of mRNAs varies considerably among species (Campagnoni and Macklin, 1988). All three size classes appear to contain mRNAs encoding both PLP and DM20 polypeptides (LeVine et al., 1990).

Expression

The *PLP/DM20* gene expresses two alternatively spliced mRNAs that encode a 30 kD (PLP) and a 25 kD (DM20) protein. In the brain, the splicing of the *PLP/DM20* gene primary transcript appears to be developmentally regulated. The developmental appearance of the DM20 protein precedes that of the myelin PLP in several species (Kronquist et al., 1987; Gardinier and Macklin, 1988; LeVine et al., 1990; Schindler et al., 1990). During the early stages of myelination the expression of the DM20 splice product predominates and it gradually declines with development. At later stages, the PLP splice product becomes the major form that is expressed.

The biological role of the DM20 protein has been unclear since its discovery over 20 years ago (Agrawal et al., 1972). Evidence that products of the *PLP/DM20* gene may be involved in processes other than myelination first came from cell biological studies on *jimpy*, a mutation in a splice site of exon 5 of the *PLP/DM20* gene (see review in Chapter 39, this volume). This *PLP/DM20* gene mutation results in a number of developmental abnormalities that occur prior to the active synthesis of myelin and which are, therefore, difficult to reconcile with a single structural role for PLP and/or DM20 in myelin.

It is becoming apparent that the expression of the *PLP/DM20* gene is not confined to oligodendrocytes in the central nervous system. To date, the expression of the gene has been reported in myelinating cells such as the oligodendrocyte in the central nervous system and the Schwann cell in the peripheral

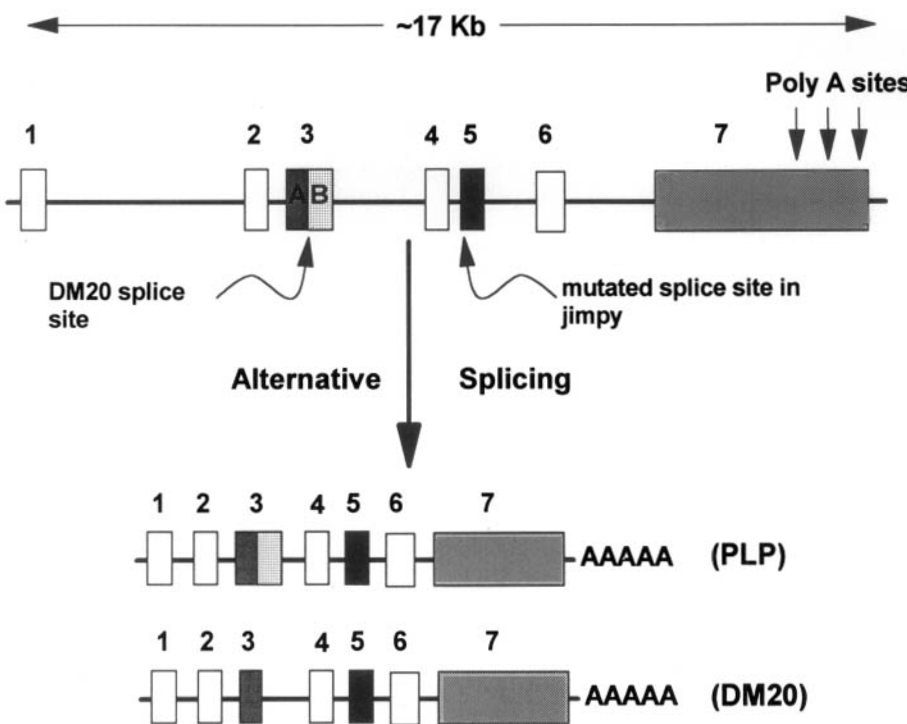


FIG. 38-3. The PLP/DM20 gene showing the alternative splicing pattern that gives rise to the PLP and DM20 mRNAs.

nervous system, although it appears that the PLP and DM20 proteins produced are not incorporated into myelin (Puckett et al., 1987; Kamholz et al., 1992). The gene is also expressed in nonmyelinating cells, such as myocardial cells in the heart (Campagnoni et al., 1992) and in cells of the embryonic nervous system (Ikenaka et al., 1992; Timsit et al., 1992), although in these cases the the DM20 mRNAs are the predominant forms of the gene that are expressed. Myocardial cells express DM20 mRNA in a postnatal developmental pattern that is roughly similar to that occurring in the brain, but these cells produce very little, if any, PLP mRNA, implying tissue-specific regulation of the splicing of the *PLP/DM20* gene primary gene product. Taken together, these results indicate that the products of the *PLP/DM20* gene play some additional role in the cell beyond that of structural proteins of the myelin sheath. It is interesting that ion channel, pore-forming properties have been ascribed to PLP-related proteins on the basis of their structure (Kitagawa et al., 1993) and that the PLP/DM20 proteins bear some homologies to ATPase (Laursen et al., 1983). These activities are consistent with the finding that DM20 mRNAs are expressed in the heart since this tissue, like the brain, contains substantial quantities of proteolipids, many of which are associated with mitochondrial function and energy transduction (Terzi et al., 1990; Hekman and Hatefi, 1991). Perhaps the role of DM20 is related to these cellular activities, but that remains to be determined.

Transcriptional Regulation

Several laboratories have attempted to analyze *cis*-acting elements and *trans*-acting factors that might regulate the activity of the *PLP/DM20* gene (Nave and Lemke, 1991; Berndt et al., 1992; Cook et al., 1992; Kim and Hudson, 1992). In studies with the rat upstream promoter region Nave and Lemke (1991) were able to identify a number of positive and negative *cis*-regulatory elements that also bound protein factors as assessed by gel retardation assays. Using the human promoter region, Berndt et al. (1992) also identified a number of elements that bound protein factors. Unfortunately, the elements identified in the two studies were not in complete agreement. In a general sense, however, both studies found that the region proximal to the transcription start site (i.e., −300 to −400 bp) was rich in *cis* elements. Both groups also found sequence motifs common to the promoter regions of many of the myelin protein genes, but these motifs were not always conserved within the same gene throughout species or among all the myelin protein genes. It seems unlikely that there is a myelin protein gene regulatory element-equivalent of the muscle-specific gene elements that bind MyoD (Weintraub et al., 1991). Recent studies by Macklin and coworkers suggest that positive regulatory elements may also reside within the first intron of the *PLP/DM20* gene (W. B. Macklin, personal communication).

2′,3′-CYCLIC NUCLEOTIDE-3′-PHOSPHODIESTERASE

CNPase activity is found in many tissues but it is particularly active in the central nervous system (reviewed by Sprinkle, 1989; Takahashi, 1992). The enzyme is localized within oligodendrocytes in the central nervous system and within Schwann cells in the peripheral nervous system. It is one of the earliest markers of cells in the oligodendrocyte lineage (Amur-Umarjee et al., 1990a), and it is widely used in cell biological studies for this purpose. Two CNPase mRNAs have been identified in rat brain and a CNPase mRNA of a different size has been identified in rat thymus (Bernier et al., 1987). Two *CNPase* genetic loci, which hybridize to the rat CNPase cDNA probes, have been reported on mouse chromosomes 3 and 11 (Bernier et al., 1988). These authors reported that the *CNPase* locus on chromosome 11 appeared to contain all the DNA sequences necessary to encode alternatively spliced CNPase mRNAs. However, it is not yet clear if both genetic loci give rise to CNPase mRNAs (perhaps in different tissues) or if the chromosome 3 locus is a pseudogene. The human gene maps to chromosome 17 (Douglas et al., 1992).

cDNAs from several different species have been isolated (Bernier et al., 1987; Vogel and Thompson, 1987; Kurihara et al., 1988) and the structures of the mouse and human *CNPase* genes have been determined (Kurihara et al., 1990; Douglas et al., 1992). They are ~6 kb and ~9 kb in length, respectively, and each consists of four exons (Figure 38-4). The mouse gene contains two transcription start sites at the beginning of the first (i.e., exon 0) and second exons (i.e., exon 1) that appear to be responsible for giving rise to two CNPase mRNAs. The mRNA produced from the upstream transcription start site (at the first exon) is smaller than the one produced from the downstream start site (at the second exon), and each encodes a separate isoform of the enzyme (Kurihara et al., 1990). The first exon splices into the interior of the second exon to produce the smaller CNPase mRNA (see Figure 38-4).

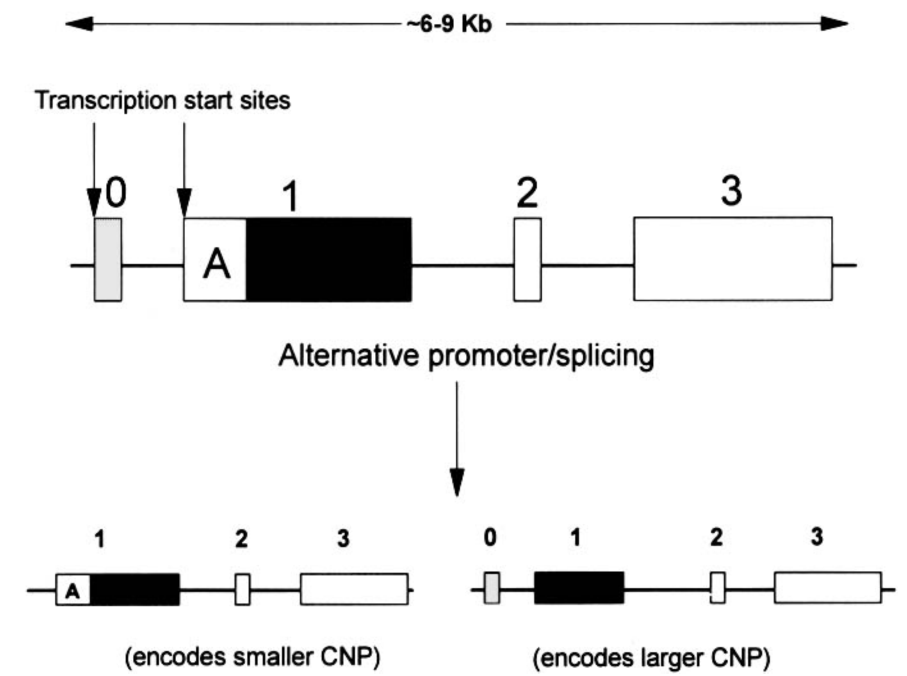


FIG. 38-4. The CNPase gene indicating the two transcription start sites at the first (exon 0) and second (exon 1) exons and the alternative splicing pattern that gives rise to two CNP mRNAs.

It has been observed that four electrophoretically separable CNPase polypeptides are expressed in the mouse (Kurihara et al., 1992). The relationship of these polypeptides to the CNPase mRNAs and the gene(s) that encode them still remains to be established.

MYELIN-ASSOCIATED GLYCOPROTEIN

MAG is the principal glycoprotein of central nervous system myelin (for recent reviews see Milner et al., 1990; and Quarles et al., 1992). The polypeptide portion of MAG consists of two isoforms with apparent molecular masses of 67 and 72 kD. On SDS gels the fully glycosylated polypeptides exhibit a molecular mass of ~100 kD. Theoretical analysis of the amino acid sequence data generated from cDNA clones indicate that MAG is a member of the immunoglobulin gene superfamily (Sutcliffe et al., 1983; Lai et al., 1987; Salzer et al., 1987). The extracellular, N-terminal domain of both MAG isoforms can be divided into five domains, ~80–90 residues in length, that have structural features similar to immunoglobulin domains, that is, a pair of cysteine residues separated by 43–62 residues (Milner et al., 1990). The data suggest that MAG is most closely related to the cell adhesion molecules N-CAM, L1, and contactin. It is generally believed that MAG is involved in some aspect of adhesion of the myelin sheath to the axonal plasmalemma. MAG has also been implicated in peripheral neuropathies in humans (Mendell et al., 1985) and in animal models (Tatum, 1993).

As shown in Figure 38-5 the MAG gene consists of 13 exons distributed over ~16 kb in the rat and has a similar structure in the mouse (Nakano et al., 1991). The gene maps to chromosome 7 in the mouse (Barton et al., 1987) and chromosome 19 in humans (Barton et al., 1987; Spagnol et al., 1989). Exons 2 and 12 are alternatively spliced to produce the two MAG mRNAs in the rat (Arquint et al., 1987; Lai et al., 1987; Salzer et al., 1987). Exons 1–4 of the gene encode 5′ untranslated region, and exons 5–9 each encode one immunoglobulin (Ig) domain, a feature common with genes of the Ig superfamily (Milner et al., 1990). The mRNA encoding the 72 kD MAG polypeptide is composed of all the exons of the gene except exon 12, and the mRNA encoding the 67 kD MAG is composed of all the exons of the gene except exon 2 (see Figure 38-5). Exon 12 contains a termination signal that creates a shorter coding region in the mRNA than would be present in its absence, thereby encoding the smaller, 67 kD MAG polypeptide. The absence of exon 2 (encoding 5′ UT region) has no effect on the size of the protein.

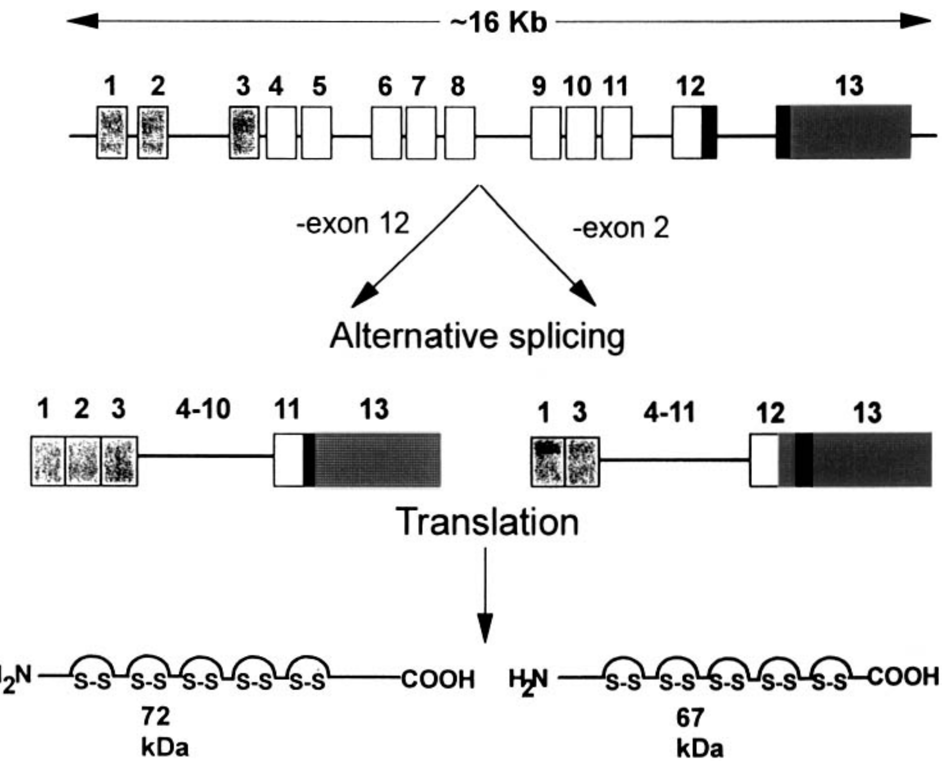


FIG. 38-5. The MAG gene showing alternative splicing to produce two mRNAs and two MAG protein isoforms. The protein diagrams emphasize the immunoglobulin domains within their structure.

MYELIN/OLIGODENDROCYTE GLYCOPROTEIN

MOG was first observed with an anonymous monoclonal antibody raised against rat cerebellar glycoproteins (Linington et al., 1984). Initially, it was thought that the smaller of the two bands (of 51 and 26 to 28 kD) recognized by the monoclonal was a breakdown product. Subsequently, however, this monoclonal was used to clone a cDNA encoding the MOG protein with a predicted size of 25 kD (Gardinier et al., 1992). On SDS-PAGE immunoblots MOG runs as a 26 to 28 kD doublet (Matthieu and Amiguet, 1990), which, on deglycosylation, runs as a single 25 kD polypeptide, leading to the conclusion that MOG polypeptides undergo at least two different types of glycosylation to give rise to two posttranslationally modified forms of the protein (Amiguet et al., 1992).

Although expression of MOG is developmentally regulated, and it appears generally with the onset of myelination, it is one of the last myelin protein genes to be expressed (Scolding et al., 1989), making it a marker of mature oligodendrocytes. A rat cDNA for MOG has been cloned, and it encodes a protein containing a signal peptide of 27 amino acids followed by a mature polypeptide of 218 amino acids (Gardinier et al., 1992). The MOG mRNA is ~1.6 kb in length. On the basis of an analysis of its predicted structure the protein contains a single glycosylation site at asn^{31} and some features that suggest it may be a member of the immunoglobulin gene superfamily (Gardinier et al., 1992).

MOG has received considerable attention because of its potential role in autoimmune-mediated demyelinating events. The protein is localized primarily at the external surfaces of the myelin sheath and oligodendrocytes, and anti-MOG antibodies can cause demyelination *in vivo* (Schluesener et al., 1987) and *in vitro* (Kerlero de Rosbo et al., 1990). Several lines of evidence suggest that MOG may be a pathogenetically important target in demyelinating conditions such as experimental autoimmune encephalomyelitis (EAE) and Multiple Sclerosis (Gunn et al., 1989; Bernard and Kerlero de Rosbo, 1991). In this regard, the M2 autoantigen described by Lebar et al. (1986), is probably MOG as assessed by their similarity in immunological cross-reactivities, cellular localization, and molecular weight (Bernard and Kerlero de Rosbo, 1991).

OLIGODENDROCYTE-MYELIN GLYCOPROTEIN

OMgp was initially discovered and isolated from the human central nervous system as a 120 kD glycoprotein that bound to peanut agglutinin (Mikol and Stefansson, 1988). OMgp is one of the "minor" protein components of myelin that appears in the central nervous system during the period of myelination. It is highly glycosylated and it appears to be specific to oligodendrocyte and myelin membranes in the central nervous system, since it was not found in Schwann cells or peripheral nervous system myelin (Mikol and Stefansson, 1988). The protein is anchored to the membrane through a glycosylphosphatidylinositol intermediate. A subpopulation of OMgp molecules from human brain appear to contain the HNK-1 carbohydrate (Mikol et al., 1990a), which has been implicated in mediating cell-cell interactions in the nervous system. A human cDNA corresponding to the OMgp mRNA has been isolated from a human fetal spinal cord library, and the primary structure of the protein has been deduced (Mikol et al., 1990b). The cDNA predicts a polypeptide sequence of 433 amino acids, including a 17-amino acid leader sequence. The size of the nascent polypeptide backbone with the leader removed is predicted to be 46 kD. Analysis of the predicted protein structure suggests the presence of four domains that includes a series of tandem leucine repeats that have been implicated in adhesive processes in other proteins. The presence of the HNK-1 carbohydrate in a subpopulation of OMgp molecules, the presence of a tandem leucine repeat domain in the predicted polypeptide sequence, and the apparent presence of OMgp at the paranodal regions of the myelin sheath have led Mikol et al. (1990b) to speculate that it functions as an adhesion molecule.

The structure and chromosomal localization of the OMgp gene has been determined (Mikol et al., 1990a). The human gene is small (~3 kb), consisting of two exons, and it maps to human chromosome 17 q11-12. An unusual feature of the OMgp gene is that it is included, along with two other unrelated genes of similar simple structure, within an intron of the neurofibromatosis type 1 gene (Viskochil et al., 1991). Interestingly, all three of these genes have a transcriptional orientation *opposite* to that of the neurofibromatosis gene (i.e., they are transcribed off the opposite strand.) At the present time, there are only a few examples in mammals of "genes within genes." It is particularly interesting that of the handful of genes that fall within this category, two are myelin protein genes, that is, *OMgp* and *MBP*.

PERIPHERAL MYELIN PROTEIN ZERO

P_0 protein accounts for over 50% of the protein in peripheral nerve myelin (Ishaque et al., 1980). The sequence has been deduced by conventional protein sequencing techniques for the bovine protein (Sakamoto et al., 1987) and from the cDNAs isolated from many species including man (Hayasaka et al., 1992), rat (Lemke and Axel, 1985), chicken (Barbu, 1990), and shark (Saavedra et al., 1989). The protein is synthesized with a signal sequence and undergoes posttranslational glycosylation. It is a transmembrane protein with a glycosylated extracellular domain, a single membrane spanning region, and a highly basic intracellular domain (Lemke and Axel, 1985). The P_0 extracellular domain has received considerable attention and analysis. It is generally thought to play an important role in the compaction of myelin through the homotypic interaction of molecules on adjacent myelin lamellae (Lemke, 1988). This notion is supported by transfection studies of P_0 cDNA constructs into cells in culture (D'Urso et al., 1990; Filbin et al., 1990). The extracellular domain is similar to an immunoglobulin variable region domain and is encoded by two exons (Lemke et al., 1988). Because most immunoglobulin-related domains in proteins within this superfamily have been found to be encoded by a single exon, it has been suggested that this partitioning between two exons is evidence for the evolution of immunoglobulin-related domains from an ancestral half-domain (Lemke et al., 1988).

The P_0 gene consists of six exons distributed over 7 kb in rats and mice (Lemke et al., 1988; You et al., 1991) and the gene maps to mouse chromosome 1 (Kuhn et al., 1990; You et al., 1991). Expression of the P_0 gene is highly restricted to Schwann cells, unlike many of the other myelin protein genes (e.g., *MBP, PLP, MAG*) that are expressed in both oligodendrocytes and Schwann cells, as well as other cell types. The elements regulating this expression appear to reside within a 1.1 kb 5′ flanking region of the gene based upon transgenic experiments (Messing et al., 1992).

SCIP

Studies on myelin protein gene expression in Schwann cells have led to the discovery of a POU domain gene, called *SCIP* (for suppressed *c*AMP-*i*nducible *P*OU), which has been proposed to act as a transcriptional repressor of myelin-specific genes (Monuki et al., 1989; Kuhn et al., 1991). *SCIP* is expressed transiently during glial development in the

central and peripheral nervous systems. In Schwann cell progenitors *SCIP* expression appears to be high at a time when expression of the P_0 and *MBP* genes is low. In contrast, when these myelin protein genes are expressed maximally, *SCIP* expression is substantially reduced. In cultured Schwann cells, agents that increase cAMP levels also induce *SCIP* expression. The *SCIP* gene has recently been isolated (Kuhn et al., 1991). It appears to be a single copy, intronless gene of approximately 3 kb in length, and it has the general features of an expressed retroposon. The gene produces a 3.1 kb mRNA that encodes a 451-amino acid, 45 to 47 kD protein, which has been localized to Schwann cell nuclear extracts by Western blot analysis (Kuhn et al., 1991).

PERIPHERAL NERVE P_2 PROTEIN

In peripheral nerve, the major proteins are P_0, MBP (referred to as P_1 in the older literature) and P_2, a basic protein distinct from the myelin basic protein. The P_2 protein was first isolated from bovine nerve roots (London, 1971) and the protein sequenced from many species (Kitamura et al., 1980; Ishaque et al., 1982; Suzuki et al., 1982). The P_2 protein has been of immunological interest because it induces experimental allergic neuritis, a demyelinating disease of the peripheral nervous system, in animals (Kadlubowski et al., 1980). The P_2 protein bears sequence homology to cellular retinol and retinoic acid-binding proteins (Crabb and Saari, 1981; Eriksson et al., 1981) and fatty acid-binding proteins (FABPs) (Veerkamp et al., 1991) and it has been shown to have a high affinity for oleic acid, retinoic acid, and retinol (Uyemura et al., 1984). cDNAs encoding the P_2 protein have been isolated and characterized from rabbit sciatic nerve (Narayanan et al., 1988) and human fetal spinal cord (Hayasaka et al., 1991) cDNA libraries. The P_2 mRNA has been detected in rabbit sciatic nerve, spinal cord, and, to a lesser extent, in brain (Narayanan et al., 1988). P_2 mRNA levels parallel myelination during development as well as the levels of microsomal enzymes involved in fatty acid elongation. These results have prompted the suggestion that the P_2 protein may be involved in fatty acid elongation, or in the transport of very long-chain fatty acids to myelin (Narayanan et al., 1988).

The structure of the mouse P_2 gene has been determined (Narayanan et al., 1991), and it appears to be similar to other members of the FABP family of genes. The P_2 gene is rather small (~4.5 kb) and consists of four exons. Phylogenetic studies suggest that the P_2 protein gene belongs to an ancient family of FABPs that diverged into two major subfamilies: one comprising the genes for mammalian liver FABP and gastrotropin, and a second comprising the genes for the retinol-binding proteins, cellular retinoic acid-binding protein, the adipocyte and heart FABPs and the P_2 protein (Medzihradszky et al., 1992). The P_2 gene is similar to the PLP/DM20 gene in that they are both members of an ancient family of proteins that have been highly conserved through evolution (Kitagawa et al., 1993).

PERIPHERAL NERVOUS SYSTEM MYELIN PROTEIN: PMP-22/PASII/SR13

Early studies indicated the existence of several glycoproteins in myelin isolated from mammalian peripheral nerve. Some of these were identified as the myelin-associated glycoprotein (Sternberger et al., 1979) and the P_0 protein (Kitamura et al., 1976). In addition to these, a very large glycoprotein of 170 kD has been described (Shuman et al., 1986) as well as low molecular weight proteins in the 19 to 23 kD range (Kitamura et al., 1976; Smith and Perret, 1986). Initially, these 19 to 23 kD glycoproteins were thought to be breakdown products of the P_0 protein (Ishaque et al., 1980) until Smith and Perret (1986) showed that a 19 kD band was not related to the P_0 glycoprotein and probably corresponded to the PAS II protein described by Kitamura et al. (1976).

Recently, two groups (Spreyer et al., 1991; Welcher et al., 1991; Snipes et al., 1992) independently cloned cDNAs from rat sciatic nerve with high homology to the growth arrest-specific mRNA, *gas 3*, and PAS II glycoprotein. The polypeptide encoded by this cDNA has been named PMP-22 and corresponds to PAS II. A human PMP-22 cDNA has also been isolated (Hayasaka et al., 1992). Like the other peripheral myelin protein genes, the expression of PMP-22 mRNA is downregulated after the nerve is crushed or transected, a fact that Spreyer et al. (1991) took advantage of to isolate their clone through differential screening techniques. The rat PMP-22 mRNA is ~1.8 kb in length and its analysis indicates that it encodes a polypeptide of 160 amino acid residues with a predicted molecular mass of ~18 kD. The polypeptide also contains a predicted leader sequence of 26 residues at its N terminus. It has four hydrophobic regions that could represent membrane-spanning domains in the molecule.

The *PMP-22* gene maps to mouse chromosome 11 and a point mutation in this gene is apparently responsible for the autosomal, dominant mutation, Trembler (*Tr*) (Suter et al., 1992a,b). In humans

the gene maps to chromosome 17 and in Charcot-Marie-Tooth 1A patients it is duplicated, thereby making it a candidate for the gene defect in these patients (Patel et al., 1992; Timmerman et al., 1992; Valentijn et al., 1992).

The rat *PMP-22* gene is developmentally regulated in the Schwann cell, its expression coinciding with myelination (Snipes et al., 1992; Spreyer et al., 1991). Its expression appears to be largely confined to the peripheral nervous system, although low levels of expression (0.1 to 0.01) can be detected in the lung, brain, and colon.

CONCLUSION

As a reading of this short review should indicate, there has been an explosion of work within the past few years on the molecular biology of myelin protein genes. The work has shown that some of the genes, such as the *MBP* and *OMgp* genes, are part of larger and more complex genetic loci. In the case of the *Golli-mbp* gene, portions of the gene are expressed in tissues outside the nervous system, including the immune system. This should encourage us to reevaluate our current concepts concerning the immunology of the myelin basic protein and the mechanisms governing its autoimmunogenicity as well as tolerance to the protein. An impressive amount of evidence has been gathered, indicating that genes we thought were prototypically nervous system-specific, for example, *MBP* and *PLP/DM20* (since myelination only occurs in the nervous system), are, in fact, expressed outside the nervous system. In the case of *PLP/DM20*, the products of the gene undoubtedly have functions completely unrelated to those of myelin structural elements, and it will be of great interest to learn what these functions are when they are elucidated. Almost all the genes encoding the "minor" myelin proteins have been cloned and molecular defects in at least one (PMP-22/PAS II) appears to result in the *Trembler* phenotype in mouse and is a strong candidate for the Charcot-Marie-Tooth 1A gene defect. The cultured Schwann cell has certainly emerged as an extremely useful cellular and molecular biological tool with which to study the regulation of myelin protein genes in the PNS. Even though the last five years have been a period of enormous growth in our understanding of the molecular mechanisms governing the expression of myelin protein genes and myelination, with all the molecular and cellular tools now available I suspect the next five years hold even more promise.

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39 Neurological mouse mutants: a molecular-genetic analysis of myelin proteins

KLAUS-ARMIN NAVE

FROM GENOTYPE TO PHENOTYPE

Genetics provides a powerful tool to dissect mechanisms of development—from genes that encode structural or regulatory proteins to complex cellular interactions. For studying mammalian development genetically, laboratory mice have become the preferred model system with their short gestation period (19 days) and generation cycle (3 months). Numerous defects of neural development have been associated with single genes in neurological mutant mice (Sidman, 1965; Hogan and Greenfield, 1984) and contributed to our knowledge of normal development. However, among the many "classical" neurological mouse mutants that occurred spontaneously, only a few have been molecularly identified, and most of these affect myelin formation. Today, the ability to experimentally generate mutations in transgenic mice of virtually any cloned gene (Mansour et al., 1988) predicts that the catalog of neurological mouse mutants will be quickly growing.

Spontaneous mutants provide the outcome of a "natural" experiment in which normal development is specifically altered. The analysis of a developmental defect such as hypomyelination, the subject of this chapter, must reach from the molecular level to the morphological and behavioral levels before a final conclusion about the defect can be made. Assuming that a neurological mouse mutant differs in only one gene function (e.g., not by a large genomic alteration that affects several genes at once), all developmental differences detectable between the normal and the mutant should be associated directly or indirectly with the normal action of the gene. However, the phenotype does not "show" the gene's normal function (or the loss of it) directly, but demonstrates how the animal develops in the absence of a specific gene function. Most organisms can respond to the deprivation of a gene function in many ways throughout development, which causes a pleiotropic phenotype by the time the observations are made.

In the simplest case, the mutation has inactivated a gene and the loss of function affects only the homozygous animal. Most genetic defects in the catalog of neurological mutant mice belong to this group of recessive mutations. In some specific cases, the mutation may have enhanced or modified the normal property of a gene in a way that the encoded protein has an altered function which also disturbs development ("gain-of-function" mutation). Heterozygous mutant mice may present a dominant or semidominant (intermediate) phenotype when one wild-type copy of the gene is not sufficient for normal development ("haploinsufficiency"). Finally, a mutant protein may have lost its own function but interferes with the activity of the wild-type protein when present in the same cell ("dominant-negative" action). When heterozygous mice with dominant mutations survive for at least 4 months and are not too handicapped to breed, these mutations can also be propagated to subsequent generations.

The identification and molecular cloning of the defective gene is a major goal but also a hurdle in the final analysis of a mutant mouse, and has been more often determined by coincidence than by the application of "reverse genetic" techniques. Once the mutation has been established at the nucleotide level, one can begin to causally link information about the wild-type gene product and its expression to the developmental processes that appear abnormal. The final conclusion of the gene's normal function is usually more difficult than anticipated due to either extensive pleiotropic action of the gene defect or to the functional redundancy built into the system. Most current research on the genetics of myelin genes focuses on this link between genotype and phenotype in myelin-deficient mouse mutants.

MUTATIONS AFFECTING MYELIN FORMATION

Myelination provides the electrical insulation of the axon by a multilayered sheath of membrane and is

the physical basis for rapid saltatory impulse conduction in the nervous system, as detailed in Section VII. The physiological requirement for myelinated axons has been met independently by the glial cells of the central and peripheral nervous system. A morphologically very similar structure, the compacted myelin sheath, is assembled by oligodendrocytes and Schwann cells, utilizing two different but overlapping sets of myelin proteins, some of which are shown schematically in Figure 39-1. This provides the opportunity to compare central and peripheral myelination in these mutants and to assess the contribution of each protein to myelin assembly.

In rodents, terminal differentiation of myelin-forming glial cells occurs largely after birth, at a time when the basic wiring pattern of the nervous system has been established. Mutations that affect myelin formation can therefore be identified by characteristic behavioral abnormalities such as shivering, ataxia, and, frequently, seizures. These signs begin when normal mice assemble large amounts of myelin, which gradually becomes a requirement for motor control and normal brain function. The specific phenotype of myelin-deficient mice necessitates also that the mutant gene is cell-type specifically expressed in Schwann cells and/or oligodendrocytes, which holds true for most (but not all) of the structural myelin protein genes (Lemke, 1988). Numerous other important molecules (cytoskeletal proteins, ion channels, cell adhesion molecules) are critical not only for myelin-forming glial cells but also other neural cell types that differentiate much earlier in embryonic development. Structural defects of these genes may have escaped detection in viable neurological mouse mutants.

Under laboratory conditions myelin is a temporarily dispensable structure. The inability of glial cells to assemble normal myelin is not immediately lethal for the animal. In fact, most mutant mice survive for several weeks after birth, which allows to compare, over a long period of time, the differentiation of normal and mutant glial cells *in vivo*. The frequently used term "*dys*myelination" denotes, in this chapter, the primary inability of the glial cell to assemble qualitative or quantitative normal myelin (the term "*de*myelination" should be reserved for the secondary loss of myelin, usually in the course of a degenerative disease such as multiple sclerosis). Most neurological mutations cause dysmyelination, but also nongenetic factors (malnutrition, poisoning) may cause dysmyelination. Conversely, some late-onset genetic disorders may present a clinical picture that includes demyelination, as in some human neuropathies of the peripheral nervous system.

In the following paragraphs, an overview is given of the molecular-genetic analysis of specific mutations in the mouse, which has improved our understanding of myelin protein function in the central and peripheral nervous system (Table 39-1). Some of these findings are directly applicable to human genetic diseases.

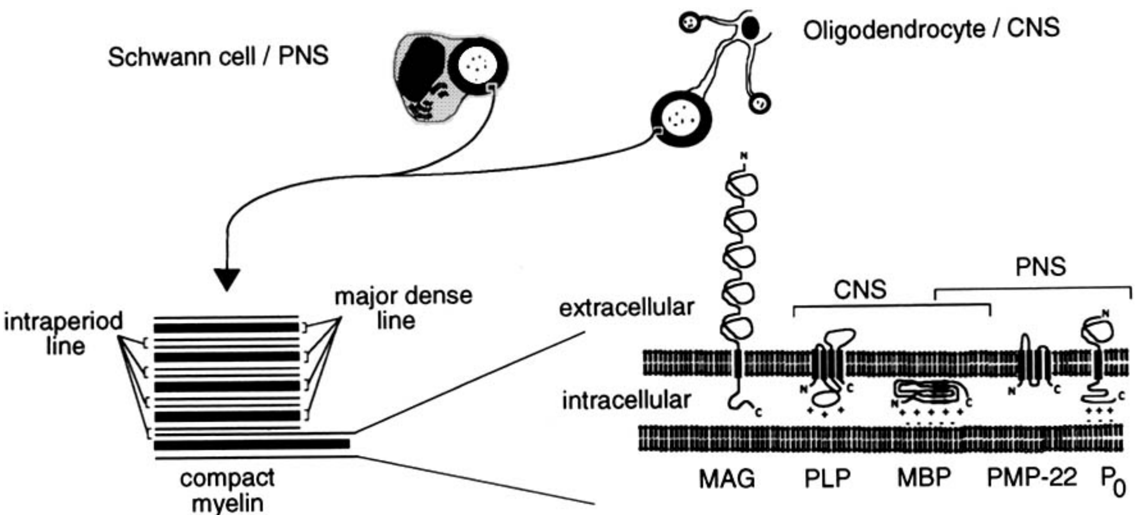


FIG. 39-1. Myelin-forming glial cells, the ultrastructure of myelin, and major proteins of the compacted membrane. Schwann cells and oligodendrocytes utilize overlapping but distinct sets of proteins to assemble myelin. Indicated are only gene products that have been implicated in the molecular defect of dysmyelinating mouse mutants: *PLP*, proteolipid protein (*jimpy*); *MBP*, myelin basic protein (*shiverer*); *PMP-22*, peripheral myelin protein (*trembler*); and P_0. In myelin of wild-type mice, the myelin-associated glycoprotein (*MAG*) is restricted to the innermost membrane facing the neuronal axon. Not shown are isoforms of PLP, MBP, and MAG derived by alternative RNA splicing.

TABLE 39–1. *Myelin Genes and Their Molecular Defect in Neurological Mutant Mice*

Myelin Gene	Mouse Mutant	Mouse Chromosome	Molecular Defect	Human Disease Equivalent	References[a]
MBP	*shiverer* *shiverer*^{mld}	18	Deletion of exons 3-7 Gene duplication with inversion of exons 3-7		Roach et al. (1985) Popko et al. (1988)
PLP	*jimpy* *jimpy*^{msd} *rumpshaker*	X	Splice defect removing exon 5, frame shift $Ala^{242} \rightarrow Val$ $Ile^{186} \rightarrow Thr$	Pelizaeus-Merzbacher disease	Nave et al. (1986) Gencic and Hudson (1990) Schneider et al. (1992)
P_0	P_0^-	1	Insertion (neo^r gene)	Charcot-Marie-Tooth disease type 1B	Giese et al. (1992)
PMP-22	*trembler* *trembler*^{J}	11	$Gly^{150} \rightarrow Asp$ $Leu^{16} \rightarrow Pro$	Charcot-Marie-Tooth disease type 1A	Suter et al. (1992a) Suter et al. (1992b)
?	*quaking*^{viable}	17	Genomic deletion ?		Ebersole et al. (1992)

[a]For additional references see text.

MYELIN BASIC PROTEIN: ABNORMAL MYELIN COMPACTION IN *SHIVERER* MICE

Myelin basic protein (MBP) comprises a complex family of related proteins, which together constitute at least 30% of the protein mass in central nervous system myelin and about 10% of peripheral nervous system myelin protein. The molecular mass of the major isoforms is 14 kD, 17 kD, 18.5 kD, 20 kD, and 21.5 kD. As described in detail in Chapter 38, the relationship of these structural proteins has been resolved by cloning of their respective cDNAs. All MBP isoforms arise by alternative RNA splicing of a primary transcript from the MBP structural gene. Inclusion of all seven exons generates the largest MBP isoform (21.5 kD), whereas the selective exclusion of exons 2, 5, and/or 6 generates the smaller isoforms (de Ferra et al., 1985; Takahashi et al., 1985). The functional difference between these proteins, however, is not known. The "myelin-specific" transcription unit of the *MBP* gene (Figure 39-2) is itself part of the considerably larger *golli-MBP* gene complex (for *g*ene in the *ol*igodendrocyte *li*neage). The upstream *golli*-specific exons encode the aminoterminal portion of MBP-like isoforms, which are expressed also outside the nervous system and which may serve functions completely unrelated to myelination (Campagnoni et al., 1993).

MBPs are positively charged membrane-associated proteins of myelin and located at the cytoplasmic surface ("major dense line") of the glial process. The function that MBP contributes to the assembly of compact myelin has been studied in the myelination defect of the *shiverer* mouse. *Shiverer* mice (genetic symbol: *shi,* mouse chromosome 18) lack any detectable MBP and were the first neurological mouse mutants successfully studied at the molecular-genetic level (Roach et al., 1983). Affected homozygous mutants fail to make normal myelin in the central nervous system and display a behavioral defect that is characteristic for *shiverer* and other myelin-deficient mice; these are best described by their given names. In the second postnatal week, *shiverer* mice develop a general body tremor, which is most pronounced when the animals initiate voluntary movements (Biddle et al., 1973; Chernoff, 1981). The shivering, which reflects a loss of spinal motor and reflex control, increases with age and is followed by seizures in the adult. Most *shiverer* mice die prematurely at about 6 months of age.

Histologically, the mutants are dysmyelinated throughout the central nervous system, whereas their peripheral myelin appears completely normal (Privat et al., 1979; Kirschner and Ganser, 1980; Rosenbluth, 1980). The ultrastructure of myelinated fibers in brain and spinal cord reveals a characteristic lack of myelin, and myelinlike structures—where present—are loosely wrapped around the axon. The intracellular adhesion zone of the extended cell process which normally forms the "major dense line" of myelin cannot be discerned. *Shiverer* oligodendrocytes appear nevertheless normally differentiated.

Biochemically, the dysmyelination is reflected in a dramatic reduction of all major myelin proteins, specifically in the complete lack of the MBPs. The latter is the primary defect of the mutant and results from a 20 kb large genomic deletion encompassing exons 3–7 of the *MBP* gene (or exons 7–11 of the larger *golli-MBP* gene) as summarized in Figure 39-2. Thus, *shiverer* mice have no coding capacity for any of the MBP isoforms (Roach et al., 1985; Molineaux et al., 1986).

FIG. 39-2. Genomic structure of the myelin basic protein locus which is partially deleted in *shiverer* mice and duplicated in the *shiverer-mld* mutant. The exons of the myelin-specific MBP transcription unit (*black boxes*) are numbered on top and the equivalent numbers of the larger *golli-MBP* gene complex are indicated below (for details see Chapter 38, this volume). The position of a genomic deletion in *shiverer* mice is indicated by a *dashed line*. Normal and antisense-containing MBP transcripts are shown by *arrows*. An inverted segment (x–x) within the duplicated *MBP* gene of *shiverer-mld* mutant is labeled *MBP-antisense*. Exon sizes are not drawn to scale.

The definitive proof that the lack of MBP itself is responsible for the dysmyelinating phenotype utilized transgenic mice. When the entire wild-type MBP gene was reintroduced into the germ line of *shiverer* mice, the tremoring phenotype dissappeared as a function of regained MBP expression (up to 25% of wild-type level) so that the mutation was phenotypically "cured" (Readhead et al., 1987). In fact, raising MBP expression levels by increasing the transgeni gene copy number in *shi/shi, shi/+,* and shi^{mld} mice resulted in myelin compaction (the formation of a major dense line) and the proportional increase in myelin thickness (Popko et al., 1987; Shine et al., 1990). This demonstrates that the amount of MBP available to oligodendrocytes is a rate-limiting step in the assembly of central nervous system myelin (Figure 39-3).

What is the functional difference of the MBP isoforms? The alternatively spliced mRNAs appear individually regulated throughout brain development, suggesting isoform-specific functions (Barbarese et al., 1978). On the other hand, it has been possible to phenotypically "cure" transgenic *shiverer* mice by reintroducing an MBP "minigene," which encodes only the smallest (14 kD) MBP (Kimura et al., 1989). Thus, the major structural function in myelin appears common to all MBP isoforms.

A *shiverer*-like phenotype has also been generated in normal mice by specifically downregulating the amount of MBP mRNA available for protein synthesis (Katsuki et al., 1988). This has been achieved by transgenic expression of the MBP gene in "antisense" orientation under the control of its cognate MBP promotor (the antisense RNA hybridizes to MBP transcripts, forming RNA duplex molecules that are degraded in the cell). Using the same technique, it may be possible to target other antisense RNAs into oligodendrocytes (or Schwann cells) of transgenic mice. This will allow to study the glial-specific function of those genes that are expressed in a variety of cell types.

The formation of antisense MBP RNA is the presumed primary defect of the mouse mutant *myelin-deficient* (symbol: shi^{mld}), an allele of the *shiverer* mutation on chromosome 18 (Doolittle and Schweikart, 1977). Here, a tandem duplication of the entire *MBP* gene, with the inversion of exons 3–7, creates a second transcription unit immediately upstream of the intact *MBP* gene (Figure 39-2), transcribing RNA that is in part complementary to the normal MBP transcript (Popko et al., 1988). The presence of this antisense RNA most likely reduces and dysregulates the amount of normal MBP mRNA toward a level insufficient for myelin formation (Fremeau

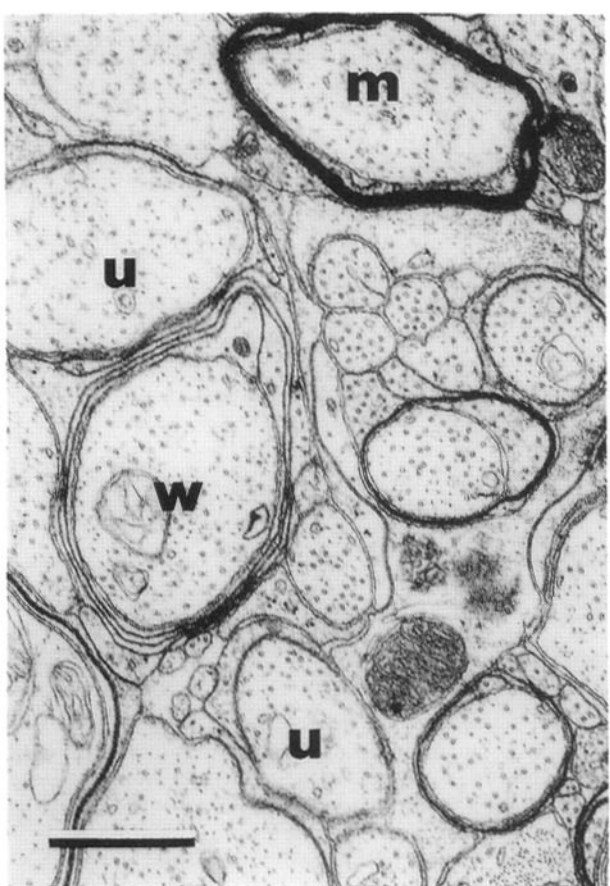


FIG. 39-3. Electron micrograph of partially myelinated fibers in the optic nerve of *shiverer* mice (*shi/shi*) transgenic for the myelin basic protein (*MBP*) gene (see Readhead et al., 1987). Whereas some axons remain unmyelinated (*u*) or loosely wrapped (*w*), more axons become ensheathed by compacted myelin (*m*) as the level of regained MBP expression increases in MBP-transgenic *shiverer* mice (here 12.5% of normal). Bar = 0.5 μm.

and Popko, 1990; Tosic et al., 1990). Morphologically, dysmyelination of shi^{mld}/shi^{mld} mice is less severe than of *shi/shi* mice. Interestingly, the genomic inversion occurred in the same region that has been deleted in *shiverer* mice, suggesting a "hot spot" of recombination in this part of the mouse *MBP* gene.

A puzzling observation in *shiverer* and its *mld* allele is the formation of functional myelin in the peripheral nervous system, including the formation of a normal "major dense line," despite the lack of MBP, which constitutes about 10% of the myelin protein made by normal Schwann cells (Privat et al., 1979; Kirschner and Ganser, 1980). Most likely, the structural function of MBP is substituted in *shiverer* by P_0, the major integral myelin protein of the peripheral nervous system (Lemke and Axel, 1985). The amino terminus of P_0 forms a "basic" domain located at the inside of the myelin membrane, which may be sufficient for the formation of a *major dense line*. Apparently, this "basic" structural function of MBP is redundant in the peripheral nervous system, even though MBP and P_0 share no obvious sequence homology (Martini et al., 1995).

PROTEOLIPID PROTEIN: DYSMYELINATION AND GLIAL CELL DEATH IN *jimpy* MICE

Proteolipid protein (PLP) is the most abundant integral membrane protein of myelin in the central nervous system, constituting roughly 50% of its protein mass. Two isoforms of PLP, the 30 kD major form (PLP apoprotein or lipophilin) and the 26 kD DM20 protein, are generated by alternative RNA splicing (Nave et al., 1987a), a recurrent feature of myelin genes. Several aspects of PLP are unusual and relevant to genetic defects associated with this gene. PLP appears relatively late in evolution as a central nervous system myelin protein of tetrapodes (Waehnelt et al., 1986), but DM20-related mRNAs exist in the central nervous system of lower vertebrates (Kitagawa et al., 1993). In higher vertebrates, PLP is specific to the compact myelin assembled by oligodendrocytes. The gene is also expressed at a low level in Schwann cells, but its function in the peripheral nervous system remains unclear. A second remarkable feature of PLP is its structural conservation in mammalian evolution. The PLP primary structure (276 amino acids) is 100% identical between mouse, rat, and humans, suggesting that PLP engages in multiple protein interactions, and, possibly, multiple cellular functions. PLP is an unusually strong hydrophobic protein with four predicted membrane-embedded domains (Figure 39-4). Alternative topological models have been proposed for PLP, but most evidence and theoretical considerations support a model with four α-helical domains and the amino- and carboxy termini oriented toward the cytoplasm, and the major dense line (Popot et al., 1991; Weimbs and Stoffel, 1992). It is likely that the membrane-associated domains of PLP interact in the lipid bilayer, possibly by forming a homooligomeric complex.

The mammalian *PLP* gene is linked to the X-chromosome, which has helped to identify its involvement in neurological defects of the mouse and in the human Pelizaeus-Merzbacher disease. These mutations have provided an unexpected glimpse at the complex function of PLP in oligodendrocyte development and myelin assembly.

In mice, three mutations of the *PLP* gene have been morphologically and genetically characterized:

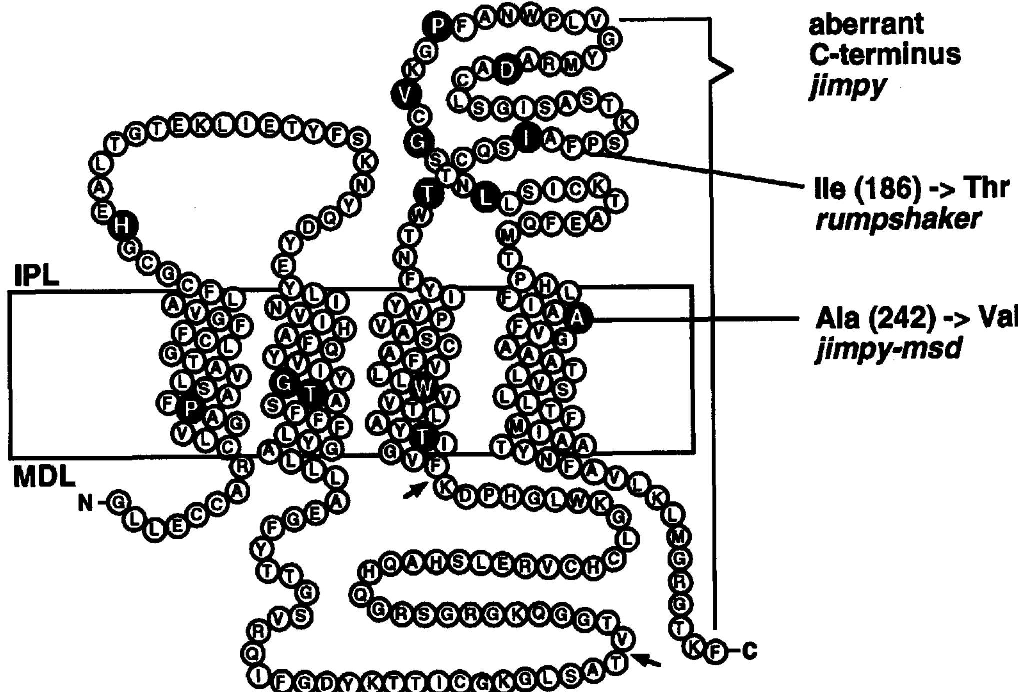


FIG. 39-4. Topological model of myelin proteolipid protein (PLP) depicted as a 4-helix bundle protein (see Popot et al., 1991) with the amino and carboxy termini oriented to the cytoplasmic surface of the lipid bilayer (*MDL,* major dense line; *IPL,* intraperiod line). The position of residues 116–150, absent from the DM20 isoform of PLP, is indicated by two *arrows.* The effect of three mutations in the mouse PLP gene (*jimpy, rumpshaker,* and *myelin-synthesis deficient*) is shown to the right. In *jimpy,* residues 207–276 are deleted and replaced by an aberrant carboxyterminus of 36 amino acids. Also indicated are the positions of single substitutions (*circles*) underlying lethal dysmyelinations in other species including the md rat (Thr74→Pro), *shaking pup* (His36→Pro), and human patients with Pelizaeus-Merzbacher disease (Pro14→Leu; Gly73→Arg; Thr155→Ile; Trp162→Arg; Thr181→Pro; Asp202→His; Pro215→Ser; Val218→Phe; Gly220→Cys; Leu223→Pro). For references see text.

jimpy (jp), myelin synthesis-deficient (*jp^{msd}*), and *rumpshaker (rsh).* All of them are point mutations that alter the structure of the encoded protein, as summarized in Figure 39-4. Each mutation results in a characteristic dysmyelinating phenotype, which is, assuming a complete loss of function in *jimpy,* more severe than the hypomyelination caused by MBP deficiency in the *shiverer* mouse.

In *jimpy* mice, the mutation is a single nucleotide change in the *PLP* gene, which inactivates the splice-acceptor site of intron 4 (the *PLP* gene spans 7 exons and is approximately 17 kb in length). This causes the loss of exon 5 from the fully spliced *jimpy* PLP mRNA with a deletion of 74 nucleotides and a shift in the normal open reading frame (Morello et al., 1986; Nave et al., 1986; Hudson et al., 1987; Macklin et al., 1987; Nave et al., 1987b). As a consequence, the encoded *jimpy* PLP lacks the last α-helical transmembrane domain, which is replaced by an aberrant carboxy terminus. The abnormally folded protein is degraded in the endoplasmic reticulum shortly after its synthesis and thus fails to reach the Golgi apparatus (Roussel et al., 1987). No immunoreactive PLP accumulates in *jimpy* oligodendrocytes and myelin membranes.

Morphologically, the central nervous system of the *jimpy* mouse is nearly completely myelin-deficient, whereas the peripheral nervous system myelin appears ultrastructurally intact (Sidman et al., 1964; Herschkowitz et al., 1971). Less than 1% of the axons normally myelinated in brain and spinal cord are ensheathed. Where present, "islands of myelin" in the mutant contain little more than a few layers of abnormally thin myelin. This myelin may consist of uncompacted whorls of membrane or display a compacted but abnormal ultrastructure: the double *intraperiod line* is fused to a single electron-dense structure, which has been attributed directly to the absence of a PLP domain from the extracellular space (Duncan et al., 1989).

The major and primary cause of dysmyelination in *jimpy,* however, is the well-documented lack of differentiated oligodendrocytes. Whereas in other myelin-deficient mutants, such as *shiverer,* oligodendrocytes are morphologically normal, in *jimpy* they remain immature. In fact, the proliferation rate of

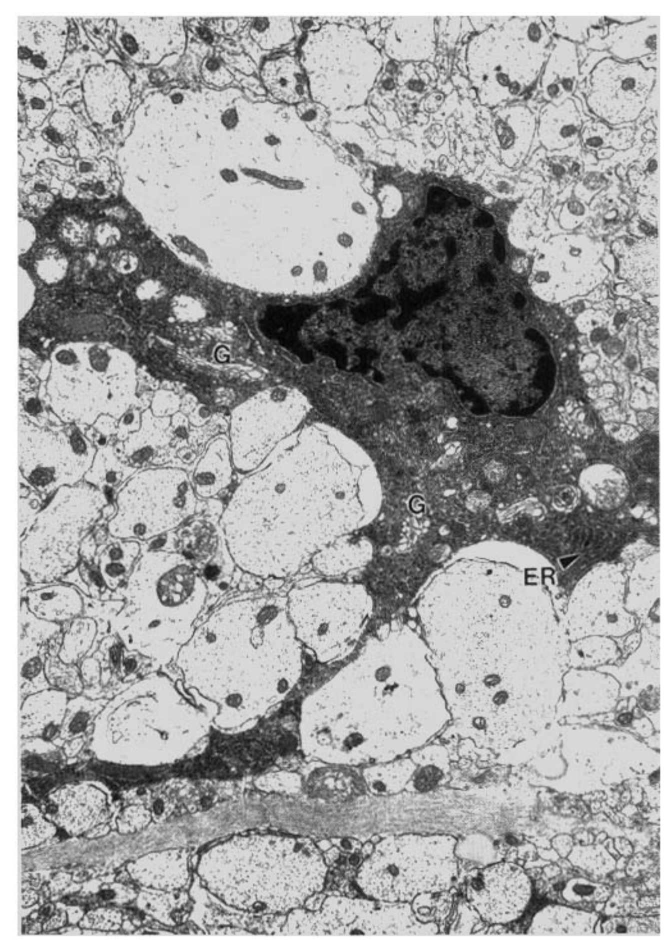
oligodendrocyte precursor cells is increased, but an abnormal rate of apoptotic cell death eliminates most of the maturating oligodendrocytes (Farkas-Bargeton et al., 1972; Skoff, 1982; Knapp et al., 1986). It appears that the normal program of oligodendrocyte differentiation comes to a halt for the vast majority of cells before myelination has started. The degeneration of *jimpy* glial cells begins even before PLP becomes immunodetectable in normal mice (Vermeesch et al., 1990). Morphologically, dying oligodendrocytes in *jimpy* show typical features of programmed cell death (apoptosis), which is part of normal glial development (Barres et al., 1992), but in the PLP mutant mice at a vastly enhanced rate (Figure 39-5A). Only few oligodendrocytes escape degeneration and developmental arrest, producing scattered "islands" of myelinated fibers. Phenotypically, *jimpy* mice are most severely affected with a general body tremor and ataxia beginning at 2 weeks of age. They remain strikingly reduced in body weight and die from seizures and convulsions in the fourth week after birth. Heterozygous *jimpy* females, which are mosaics with respect to the X-linked *PLP* gene are behaviorally normal.

The phenotype of the allelic mouse mutant *jimpy*^{msd} (for myelin synthesis-deficient) is very similar to *jimpy*. Compared to *jimpy*, about twice as many glial cells escape premature degeneration and form visible myelin structures with a similar ultrastructural defect (Billings-Gagliardi et al., 1980). Remarkably, the molecular defect of *jimpy*^{msd} is a conservative amino acid substitution (Ala^{242}→Val) within the last transmembrane domain of PLP (Gencic and Hudson, 1990). This mutant protein is apparently also unstable, as very little of it is incorporated into myelin (Nussbaum and Mandel, 1973). It is unclear in which way this subtle structural change causes a dramatic loss of protein function, but it points again to critical protein interactions of PLP in the plane of the membrane.

A novel mutation of the *PLP* gene presents the mouse mutant *rumpshaker* (Schneider et al., 1992). Like the allelic mutants *jimpy* and *jimpy*^{msd}, the molecular defect is a single amino acid substitution of PLP (Ile^{186}→Thr); however, the phenotype of the mouse is different. *Rumpshaker* mice have more myelin than other dysmyelinated mutants and the degree of dysmyelination varies more strikingly between central nervous system regions than in other PLP mutants. Early myelinated spinal cord axons are nearly normally ensheathed, whereas late myelinated areas, such as the optic nerve, remain severely hypomyelinated. Ultrastructurally, oligodendrocytes appear differentiated and, most importantly, most of them escape apoptotic cell death. Thus, *rumpshaker* mice have a normal complement of mature oligodendrocytes (Griffiths et al., 1990). The mutant DM20 isoform (but not PLP itself) can be readily detected in the myelin membrane with site-specific antibodies (Schneider et al., 1992). This suggests that the *rumpshaker* mutation interferes with the oligodendrocytes ability to deposit PLP in the myelin membrane, and that the surface expression of DM20 may be sufficient to allow oligodendrocytes to survive (Figure 39-5b). *Rumpshaker* mice are viable, live without seizure activity, and have a normal life span.

In several other mammalian species, mutations of the *PLP* gene have been identified, and most of them result in a structurally altered form of PLP and DM20 (Figure 39-4). In a myelin-deficient strain of the rat (*md*), a point mutation changes Thr^{74}→Pro in the second transmembrane domain of PLP (Boison and Stoffel, 1989). A single base change in the canine *shaking pup* causes the substitution His^{219}→Pro close to the first transmembrane domain of PLP (Nadon et al., 1990). Ultrastructurally, the oligodendrocytes in both mutants show unusual intracellular vacuoles, presumably caused by aberrantly folded PLP unable to exit the endoplasmic reticulum. Mutations of the human *PLP* gene, including the complete deletion of the locus, have been identified in several patients with Pelizaeus-Merzbacher disease (Pham-Dinh et al., 1991).

When the entire normal *PLP* gene was reintroduced into *jimpy* mice as an autosomal transgene (in an attempt to restore myelination), the wild-type *PLP* transgene was normally expressed but the dysmyelinated phenotype of the *jimpy* mice did not change. Thus, in experimentally created "heterozygous" oligodendrocytes (normal heterozygous *jimpy* females are mosaic with respect to the X-linked *PLP* gene), the *jimpy* allele acts in a dominant-negative fashion (Schneider et al., 1995). This may reflect that normal and mutant proteins interact in the endoplasmic reticulum, form aberrantly folded complexes, and are, in part, jointly degraded by resident proteases. Surprisingly, PLP-transgenic wild-type mice which overexpress the normal *PLP* gene more than twofold become themselves myelin-deficient with tremors, seizures, and premature death (Kagawa et al., 1994; Readhead et al., 1994). The extent of dysmyelination in the PLP transgenic mice and their survival time is determined by the degree of PLP overexpression (e.g., the transgene copynumber). Thus, both PLP overexpression and structural mutations interfere in a similar way with myelin formation; however, the mechanisms that make oligodendrocytes so vulnerable to and dependent on PLP function are not understood.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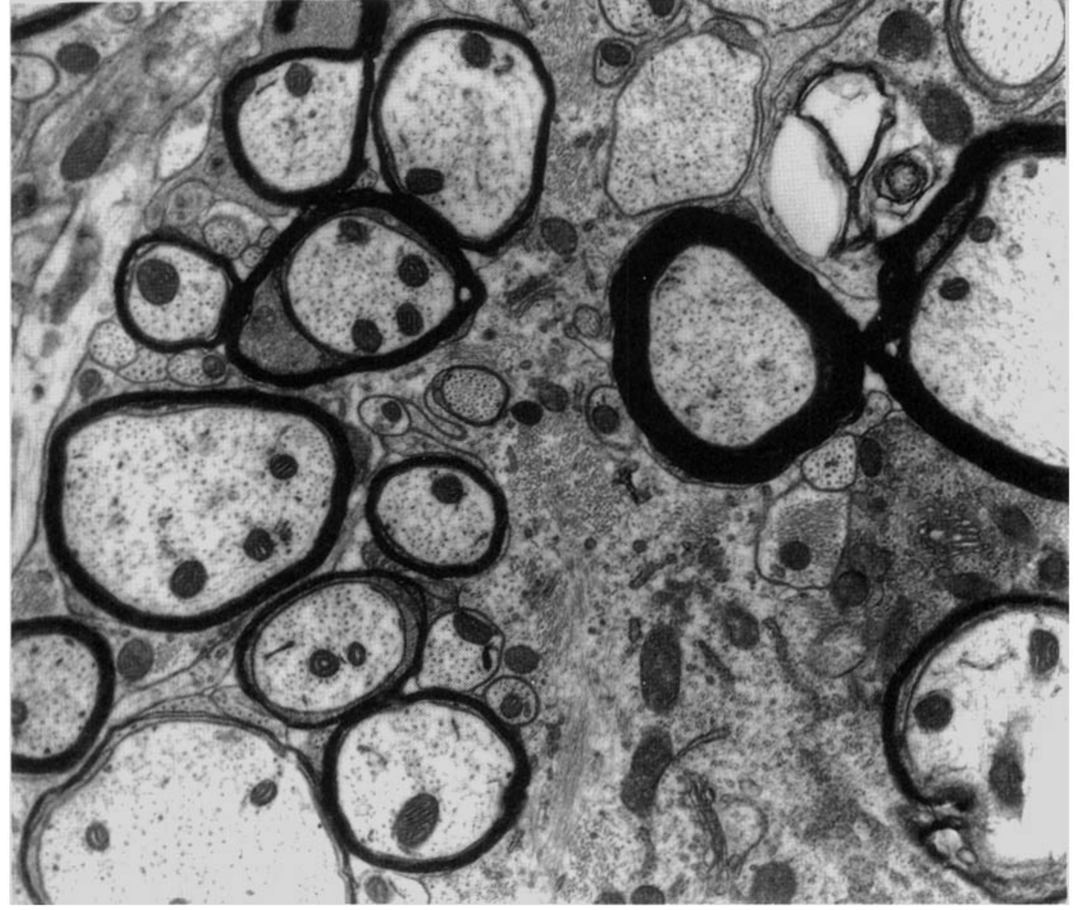


PROTEIN ZERO: AN ADHESION MOLECULE OF SCHWANN CELLS AND P_0-DEFICIENT MICE

In myelin of the peripheral nervous system, the major protein component—approximately 50% by mass—is protein zero (P_0), a glycosylated membrane protein of 219 amino acids (molecular weight: 30 kD). With a single transmembrane domain and one immunoglobulin (Ig)-like extracellular domain, P_0 is an ancestral member of the Ig superfamily of proteins (Lemke and Axel, 1985; Lemke et al., 1988).

The presence of ultrastructurally normal peripheral nervous system myelin in the myelin basic protein-deficient *shiverer* mouse had provided indirect evidence that P_0 serves at least one function related to that of myelin basic protein in the central nervous system: mediating the intracellular adhesion of two apposed myelin membranes with formation of the major dense line. This redundancy in function with myelin basic protein would be accomplished by the basic intracellular domain of P_0. The second and presumably major function of the protein concerns its extracellular Ig domain oriented toward the intraperiod line of myelin. Here, the direct implication of P_0 as a homophilic cell adhesion molecule resulted from transfection studies and ectopic surface expression of P_0 in nonglial cells: P_0 mediates both the formation of junction structures between cells in a monolayer and the aggregation of cells in suspension (D'Urso et al., 1990; Filbin et al., 1990; Schneider-Schaulies et al., 1990). That P_0 is essential for the formation of peripheral myelin was first demonstrated by the expression of P_0 antisense RNA in cultured Schwann cells (Owens and Boyd, 1991), which interfered with the normal assembly of myelin. Without a known spontaneous mutation of the P_0 gene (located on mouse chromosome 1), the *in vivo* analysis of P_0 function had to await the targeted disruption of the gene in transgenic mice.

P_0-deficient mice have been generated by homologous recombination of the P_0 gene in mouse embryonic stem cells with the cloned gene (interrupted by the neomycin resistance gene in exon 1) and subsequent generation of germline chimeric mice (Giese et al., 1992). Heterozygous animals lacking one functional copy of the P_0 gene in the F1 generation are phenotypically normal, but homozygous P_0-deficient mice (P_0-/P_0-) develop a novel behavioral phenotype shortly after the second postnatal week: a body tremor and dragging movements of the hindlimbs. Seizures and paralysis are absent, however, and the mutants have a normal life span. As predicted, the underlying defect is the inability of Schwann cells to assemble a multilayered normally compacted myelin sheath. The events preceding myelin formation, including the association of Schwann cells with single axons in a 1:1 ratio are not disturbed. Interestingly, Schwann cells in one and the same mutant animal differ considerably in the degree of dysmyelination, even though they are genetically identical. It is thus likely that the complete loss of P_0 function is, at least in part, masked by other variables, in particular, some cell adhesion molecules (myelin-associated glycoprotein; N-CAM), which are abnormally abundant in the myelinlike ensheathements of P_0-deficient mice. Even PLP, which is normally expressed at a very low level in Schwann cells and which is excluded from peripheral myelin, becomes a prominent myelin protein in the absence of P_0. Future experiments will define the direct and indirect effects of this novel mutation, including the intriguing finding of many degenerating axons engulfed by P_0-deficient Schwann cells.

It is interesting to compare the loss of P_0 in mutants with the ablation of Schwann cells in transgenic mice when coupled to the onset of P_0 expression. Using the promoter and regulatory region of the P_0 gene in a fusion gene construct, a bacterial toxin has been experimentally expressed in Schwann cells of transgenic mice (a few molecules of the diphtheria toxin A peptide are sufficient to kill a cell). This dominant "mutation" in mice causes the physical loss of the majority of myelin-forming Schwann cells, which begin to express P_0 after associating in a 1:1 ratio with axons (Messing et al., 1992). The behavioral phenotype of the Schwann cell-ablated mice closely resembles that of P_0-/P_0- mutants. Indirect effects of this lesion include the proliferation of non-myelin-forming Schwann cells, presumably triggered by the myelin-deficient local milieu, and skeletal muscle atrophy. The abnormal degeneration of large caliber axons, noted in the peripheral nervous system of P_0-deficient mice, is less prominent.

FIG. 39-5. (A) Electron micrograph of a dying oligodendrocyte in the spinal cord of a 3-week-old *jimpy* mouse carrying a mutation in the proteolipid protein gene. Note the chromatin condensations characteristic of apoptosis (programmed cell death) and the complete absence of myelin from surrounding axons (*G*, Golgi apparatus; *ER*, endoplasmatic reticulum). (B) Hypomyelination in the spinal cord of *rumpshaker* mice. This mutation is allelic to *jimpy* but differs by the survival rate and number of mature oligodendrocytes. The proximity of myelinated and dysmyelinated large-caliber axons is striking and indicates a defect in terminal oligodendrocyte differentiation rather than a structural defect of PLP-dependent myelin assembly.

The "mutant" mice die at 3 to 4 months of age, but the exact cause of death has not been determined.

PMP-22: A SCHWANN CELL GROWTH DEFECT IN *trembler* MUTANT MICE

A second integral membrane protein specific to Schwann cells is the glycoprotein PMP-22 (for 22 kD peripheral myelin protein). Only recently discovered as a gene product that is differentially regulated in Schwann cells proximal and distal to a peripheral nerve cut, PMP-22 has been quickly recognized as a myelin protein, which is important for normal Schwann cell development (Spreyer et al., 1991; Welcher et al., 1991).

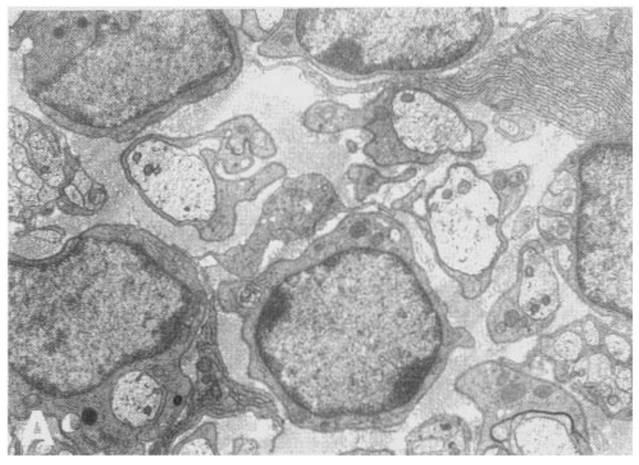
The primary structure of the 160-residue PMP-22 protein is well conserved between rodents and human (87%), in particular within the four predicted transmembrane domains. PMP-22 is identical to the gas-3 protein, which was identified first as a growth arrest-specific gene transcript in NIH3T3 cells (the reason for the expression of this myelin gene in a fibroblast cell line is unclear). The glial protein is incorporated into compacted myelin and is coregulated with the major myelin genes of the peripheral nervous system in both development and, after injury, in the regenerating nerve. PMP-22 could be a structural component of myelin, but a nonstructural role in Schwann cells is also likely, for example, in maintaining the quiescent state, but this has not been shown experimentally. Some insight into PMP-22 function came from two neurological mouse mutants and the involvement of the *PMP-22* gene in a human peripheral neuropathy (see the subsection *Charcot-Marie-Tooth Disease* this chapter).

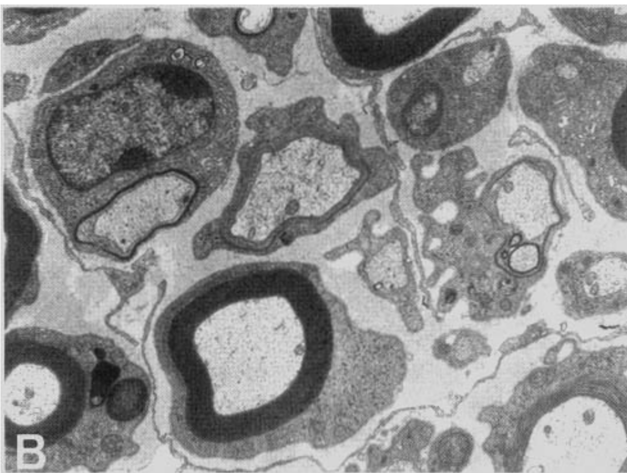
A mutation of the *PMP-22* gene is the primary cause of dysmyelination in the *trembler* mouse (Tr; mouse chromosome 11), which has a myelination defect restricted to the peripheral nervous system (Falconer, 1951). A point mutation has been identified that causes a nonconservative amino acid substitution (Gly^{150}→Asp) within the predicted fourth transmembrane domain of PMP-22 (Suter et al., 1992a). Most likely the mutant protein is abnormally folded and nonfunctional. The *trembler* mutation is semidominant, which implies that either a 50% reduction of functional PMP-22 is not tolerated (haploinsufficiency) or that the mutant protein exerts a dominant-negative effect on the normal protein (as observed in PLP-transgenic *jimpy* mice).

Two aspects of the *trembler* phenotype are important to the normal cellular PMP-22 function. First, the majority of large caliber axons in the sciatic nerve remains completely naked (up to 70% in Tr/+ mice instead of 1% in normal mice), and the myelin sheaths, where present, are abnormally thin and compacted (Henry and Sidman, 1988). Second, myelination is arrested in affected Schwann cells at the promyelin stage when they have segregated with axons in a 1:1 ratio. At the same time, the total number of Schwann cells in the *trembler* peripheral nerve is dramatically increased relative to normal. This could be either the primary defect (in a growth-arrest function of PMP-22) or a less specific secondary response to dysmyelination (as seen in Schwann cell-deficient mice). Older *trembler* mice show signs of both dysmyelination and demyelination with characteristic "onion bulb" formations, a sign of Schwann cell hypertrophy related to the pathology of peripheral nerves in patients with Charcot-Marie-Tooth disease.

Homozygous *trembler* mice (*Tr/Tr*) assemble virtually no peripheral myelin. The behavioral phenotype of hypomyelinated *Tr/+* and myelin-deficient *Tr/Tr* mice is nevertheless the same: a coarse action tremor that begins at the end of the second postnatal week and results in moderate quadriparesis and an overall waddling gate. Interestingly, *trembler* mutant Schwann cells exert secondary effects on the engulfed nerve axons: their diameter is significantly decreased, the axonal transport is slowed down, and neuronal filaments are abnormally phosphorylated; however, the nature of this glial-neuronal interaction is not understood (Aguayo et al., 1977; de Waegh et al., 1992). Remarkably, all *trembler* mice, including the homozygous, are fully viable and long-lived, suggesting that under the artificial living conditions of the laboratory cage, a mouse can survive in the complete absence of peripheral myelin (Henry and Sidman, 1988). This contrasts with the lethal phenotype of a second *trembler* allele and the limited life span of Schwann cell-deficient mice.

The second mutation of the PMP-22 gene was identified in *trembler-J* (Tr^J) mice. In this allele, a nonconservative substitution (Leu^{16}→Pro) alters the first transmembrane domain of PMP-22 (Suter et al., 1992b). As in *Tr*, the mutant protein is presumably nonfunctional and aberrantly folded. The phenotype of heterozygous $Tr^J/+$ mice is similar to the original *Tr* allele; however, the expression is more clearly semidominant: a marked decrease of peripheral myelination is associated with a wide spectrum of Schwann cell differentiation, many cells being arrested at the "promyelin" stage (Figure 39-6). Tr^J/Tr^J mice, with a complete lack of peripheral myelin, are clinically more severely affected (than the viable *Tr/Tr*) with quadriparesis and premature death at about 3 weeks of age. The latter is surprising, considering that the homozygous animals of both *trembler* alleles appear equally myelin-deficient, but only





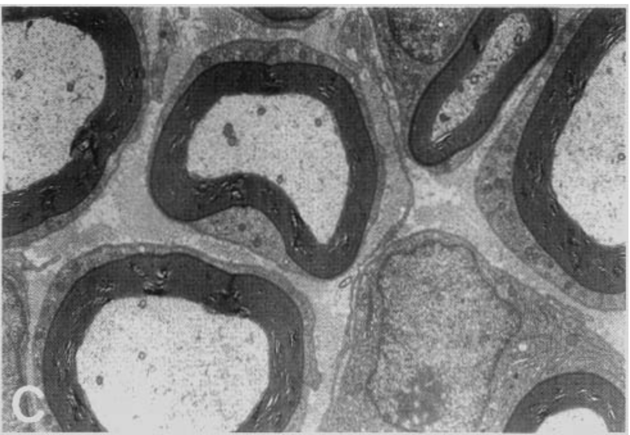


FIG. 39-6. Transverse section of sciatic nerves from 13-day-old *trembler*^{J} mice with a point mutation in the gene encoding PMP22. (A) In homozygous animals (Tr^J/Tr^J), myelin is completely lacking, but individual axons have been engulfed by mutant Schwann cells in a 1:1 ratio. (B) In heterozygous mice ($Tr^J/+$), many cells are arrested at the "promyelin" stage, but there is a wide range of Schwann cell differentiation (C) when compared to sciatic nerves from unaffected littermates. [Adapted from Henry and Sidman (1983), with permission.]

trembler^J mice succumb to it. In fact, when the amount of MBP in sciatic nerve extracts is taken as a measure of dysmyelination, mice of the lethal *trembler-J* allele are better myelinated than the viable *trembler* allele (Fryxel, 1983). This paradox could result from a different genetic background of the two mutations or relate to the expression of PMP-22 in other parts of the nervous system. Whether the increased proliferation of Schwann cells in either *trembler* mutant prevents normal myelination or, vice versa, is a response to dysmyelination is not known. Experiments involving transgenic mice and ectopic PMP-22 expression in cultured cells may help to define the function of this protein during growth and differentiation.

OTHER MYELIN GENES

Several neural genes which have been molecularly cloned qualify as "myelin genes" because they either encode structural proteins of the myelin sheath or myelin-associated regulatory molecules, such as transcription factors. Intensively studied proteins in this group include the myelin-associated glycoprotein (MAG), myelin-oligodendrocyte glycoprotein (MOG), the enzyme 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNPase), or the POU-homeobox protein SCIP (suppresed *c*AMP-inducible *POU*). So far, these proteins have not been correlated with existing neurological mutations in the mouse. However, it will be possible to study all of these genes as a loss-of-function mutation in the near future. Possibly, some of these engineered mutations may not simply reveal the proteins normal function, as the phenotype is too complex, embryonically lethal, or because there is no detectable phenotype at all (if other genes serve redundant functions). There are multiple examples for each scenario in other systems. The second group of "myelin genes" is at present only genetically defined with a phenotype that affects primarily myelination. A well-studied gene in this group is *quaking* (*qk*), a recessive mutation on mouse chromosome 17 (Sidman et al., 1964). Homozygous mice carrying the viable *quaking* allele (qk^v/qk^v) show the typical behavioral signs of dysmyelination, but rarely seizures, and have a normal life span. The myelin deficiency of the central nervous system is more severe than the dysmyelination of the peripheral nervous system, suggesting (but not proving) that the *qk* protein is predominantly expressed in brain and spinal cord. Axons are thinly myelinated, but rarely naked. In peripheral myelin of *quaking*, a lack of normal compaction and enlarged "intraperiod line" of some myelinated fibers has been noted (Trapp, 1988). My-

elin-associated glycoprotein (MAG), which is normaly restricted to the innermost myelin layer facing the axon is distributed throughout the compact myelin sheath of *quaking* mice. Such an ultrastructural defect may be caused by a dysregulation of the alternative MAG mRNA splicing, whereas the *MAG* gene itself (mapped to mouse chromosome 7) cannot be the site of the mutation. Homozygous male mutants are sterile (caused by a sperm defect), which indicates that the *qk* gene serves a second function in testes or that the viable qk^v mutation affects more than one gene. The latter is supported by the generation of several *qk*-allelic point mutations by saturation mutagenesis—some of which are embryonically lethal. The recent finding of a large genomic deletion associated with chromosome 17 of qk^v mice (Ebersole et al., 1992) suggests that the *quaking* gene will soon be identified.

IMPLICATIONS FOR HUMAN GENETIC DISEASES

Neurological mouse mutants may provide true animal models for human neurological diseases. Three myelin genes have been specifically implicated in homologous genetic disorders in man and mouse: the *PLP* gene in patients with Pelizaeus-Merzbacher disease, the *PMP-22* gene in patients with Charcot-Marie-Tooth disease type 1a, and the P_0 gene in patients with Charcot-Marie-Tooth disease type 1b. In two cases the neurological mouse mutants (*jimpy, trembler*) were instrumental as models for the identification of the human gene defects.

Pelizaeus-Merzbacher Disease

Pelizaeus-Merzbacher disease (McKusick No. 312080) is a lethal X-chromosome-linked disorder characterized by the lack of myelin formation in the central nervous system. Based on the degree of myelin deficiency and the onset of clinical symptoms, several subtypes of Pelizaeus-Merzbacher disease have been defined. Clinically, Pelizaeus-Merzbacher disease is diagnosed when male infants (*connatal* type) or young boys (*classical* type) develop a severe defect of motor control (nystagmus, spasticity, ataxia) and often psychomotor retardation as a result of a generalized myelin deficiency of the central nervous system (as diagnosed by magnetic resonance imaging). The homology of this genetic disease with the dysmyelinating phenotype of the X-chromosome-linked mouse mutants (Koeppen et al., 1987) and the identification of point mutations in the rodent *PLP* gene prompted the detailed analysis of the human gene in Pelizaeus-Merzbacher disease patients (Hudson et al., 1989). As of today, more than thirty different mutations of the *PLP* gene have been associated with Pelizaeus-Merzbacher disease (Figure 39-4), including the complete deletion of the *PLP* locus (Gencic et al., 1989; Troffater et al., 1989; Pham-Dinh et al., 1991; Raskind et al., 1991; Iwaki et al., 1993). Most of the *PLP* gene mutations are single-base changes that cause amino acid substitutions of the protein. As in the mouse models of Pelizaeus-Merzbacher disease, it is likely that the onset of the disease and its clinical course is determined by the specific functional defect of the mutant PLP. The abnormal regulation of the *PLP* gene may also cause Pelizaeus-Merzbacher disease, because an interstitial duplication of the human X chromosome which includes the *PLP* locus results clinically in Pelizaeus-Merzbacher disease (Cremers et al., 1987), and a mere twofold overexpression of the normal *PLP* gene causes dysmyelination in transgenic mice (Readhead et al., 1994).

Charcot-Marie-Tooth Disease

Among the human motor-sensory neuropathies, Charcot-Marie-Tooth disease type 1a (Charcot-Marie-Tooth1a or human motor-sensory neuropathy1) is the most common inherited peripheral neuropathy, linked to human chromosome 17 (autosomal-dominant). Clinically, nerve conduction velocities are decreased and associated with sensory losses and muscle weakness as the first signs of the disease. Schwann cells are hypertrophic with ultrastructural signs of dys- and demyelination ("onion-bulb formation"). As in Pelizaeus-Merzbacher disease, mutations affecting the structure of the protein and those affecting expression level cause a very similar disease. Cytogenetic evidence indicated that in several families Charcot-Marie-Tooth1A is associated with an interstitial duplication on the short arm of chomosome 17. Human chromosome 17 is, to a large extent, syntenic to mouse chromosome 11 which encodes the peripheral myelin protein PMP-22. The human *PMP-22* gene is contained within the duplicated part of chromosome 17 (Patel et al., 1992; Timmerman et al., 1992) suggesting that 50% overexpression of the *PMP-22* gene causes Charcot-Marie-Tooth1A. Other families were identified with point mutations in the *PMP-22* gene. In fact, the identical mutation identified first in the mouse mutant *trembler-J* ($Leu^{16} \rightarrow Pro$) was found in a human patient with Charcot-Marie-Tooth1a (Valentijn et al., 1992). This also demonstrates that the clinical picture of the same molecular defect can be quite different between a short-lived mouse and a human patient. A partial deletion of chromosome 17 (the reciprocal meiotic loss of the

interstitial duplication in Charcot-Marie-Tooth1a patients) was identified in families with the peripheral myelin disease hereditary neuropathy with liability to pressure palsies (McKusick 162500) (Chance et al., 1993). This latter disease affects young adults and is defined by a recurrent demyelinating condition of peripheral nerves following minor traumas, most likely caused by haploinsufficiency of the human *PMP-22* gene. The pathomechanisms, however, remains to be determined. A second familial form of Charcot-Marie-Tooth disease (type 1B) is linked to human chromosome 1 and most likely caused by mutations in the gene for P_0 (Hayasaka et al., 1993; Kulkens et al., 1993). The autosomal-dominant mode of inheritance contrasts, at first glance, with the previous observation that heterozygous P_0- mice are normally myelinated, but may be explained with a dominant-negative function of mutant P_0 in compacted myelin. A complete loss of P_0 or PMP-22 function may underlie the rare Déjerine-Sottas neuropathy.

CONCLUSIONS

The combination of classical and molecular genetic techniques has provided insight into the *in vivo* function of the major myelin protein genes. Most dysmyelinating mouse mutants have been studied in detail without prior knowledge of the underlying primary genetic defect which allowed an "unbiased" analysis of each phenotype. In general, the developmental abnormality of the mutant mice is in good agreement with the predicted role of the major structural myelin proteins in assembly and maintaining the multilayered compacted sheath. A certain degree of redundancy between functionally related proteins may mask the full loss of function of a single gene. For some myelin proteins, a more complex phenotype has provided a glimpse at cellular functions of myelin proteins, which may exceed that of simple structural components. It is possible that these myelin proteins have been recruited as structural components from a primary function in glial cells, which is yet unrelated to myelin assembly. In the near future, the number of available myelin mutants will increase as more genes have been molecularly cloned and are used to generate transgenic mouse mutants. Further unraveling of the myelination process may soon come to illustrate that mammalian brain development can be dissected with the tools of genetics.

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Note added in proof: Target inactivation of the mouse MAG gene has now been reported by Montag et al. (1994) and Li et al. (1994).

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40 Axoglial interactions at the cellular and molecular levels in central nervous system myelinated fibers

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It is now clear that myelinated fibers and nodes of Ranvier in the central nervous system are complex structures where the axon, the oligodendrocyte, and the perinodal astrocyte interact in a highly coordinated manner. In this chapter we discuss the interaction of axons, oligodendrocytes, and astrocytes in the maturation of the central myelinated fibers and node of Ranvier. In examining the collaboration between these three cell types in myelinated central nervous system axons, we focus on three fundamental questions.

First, what are the factors that control the development of the myelinated fiber at the *cellular* level and the mechanisms that determine the structure of the myelin sheath (and thus of its component spiral of oligodendroglial membrane? In most myelinated tracts the internodes have properties that optimize a number of axonal functions (Rushton, 1951). One example is provided by the thickness of the myelin sheath. Conduction velocity scales linearly with diameter in myelinated fibers and is maximized when myelin thickness has a value such that the g ratio (diameter of axon within myelin sheath: outer diameter of myelin sheath) is close to 0.6. The number of myelin lamellae in most central nervous system myelinated fibers is matched to axon diameter so that g ratios fall close to this optimal value over a wide range of diameters (Waxman and Bennett, 1972). A second example is provided by the internode distance, or distance between the nodes of Ranvier. Physiological studies show that there is an optimal ratio of internode distance to fiber diameter (approximately 100 to 200), which maximizes conduction velocity (Brill et al., 1977). In many white matter tracts [excluding "delay line" tracts where axons interpose specific time-delays (Waxman, 1971) and preterminal axons where short myelin internodes mediate impedance matching (Waxman and Brill, 1978)], the measured values for internode distance are close to those predicted for maximization of conduction velocity (Hess and Young, 1952; McDonald and Ohlrich, 1971). This optimization, in terms of the structure of the myelinated internodes, focuses attention on the interactions between axons and glial cells that regulate parameters such as the number of lamellae in the myelin sheath and the length of the oligodendroglial wrapping, which determines internode distance.

Second, what is the *molecular* architecture of central nervous system myelinated fibers, how are the ion channels distributed in the axon membrane, and how do glial-axonal interactions localize channels at specific sites? The voltage-gated Na^+ channels that underlie the depolarization phase of the action potential are not distributed at random along the myelinated axon but, on the contrary, are clustered in high densities in the axon membrane at the node of Ranvier, precisely where they are required for action potential electrogenesis (Ritchie and Rogart, 1977; Waxman, 1977). "Fast" K^+ channels, in contrast, are localized in the axon membrane under the myelin in the paranodal and/or internodal regions (Chiu and Ritchie, 1981b; Kocsis et al., 1982). A detailed account of the distribution and function of these, and other, ion channels and other active molecules in the membrane of the myelinated fiber, can be found in Waxman and Ritchie (1993). The clustering of ion channels at specific sites in the axon membrane, precisely correlated with the location of the myelin sheath and the node of Ranvier, raises the question of how interactions with glial cells modulate the molecular structure of the axon membrane.

Third, what is the relationship between perinodal astrocyte processes and the node of Ranvier? Perinodal astrocytes, as well as axons and oligodendrocytes, are major components of central nervous system white matter tracts. Suzuki and Raisman (1992) have proposed a model in which longitudinally oriented oligodendrocyte processes and radially oriented astrocyte processes interdigitate in a stereotyped manner within central nervous system white matter. Electron microscopy has, in fact, demon-

strated a highly specific relationship between astrocyte processes and the node of Ranvier in the central nervous system.

The node of Ranvier in the central nervous system comprises three cell types: the axon, the oligodendrocyte, and the astrocyte. In this discussion of central nervous system myelinated fibers, we examine interactions not only between the oligodendrocyte and the axon, but also with the astrocyte.

LOCAL AXON-OLIGODENDROCYTE INTERACTIONS REGULATE MYELIN FORMATION

Oligodendrocytes are the cells of origin of central nervous system myelin sheaths (Bunge et al., 1961; Hirano, 1968; Waxman and Sims, 1984). At early stages of development the axon is loosely ensheathed by processes arising from immature, relatively undifferentiated glial cells (Figure 40-1). This early ensheathement soon gives way to a spiral wrapping of the axon by oligodendroglial processes that form compact myelin. In some tracts the immature myelin sheaths are initially close to the oligodendroglial cell body (Remahl and Hildebrand, 1990), but, as maturation proceeds, the sheaths appear to be displaced radially; thus, in many mature white matter tracts, the myelin sheaths remain connected only by thin cytoplasmic bridges to the oligodendroglial cell bodies from which they originate. This morphological arrangement is illustrated at the light microscopic level in Figure 40-2, which shows a dye-filled image of a presumed oligodendrocyte from the intact rat optic nerve (Butt and Ransom, 1989). The number of axons, myelinated by a single oligodendrocyte, ranges from less than five for some oligodendrocytes to dozens for others (Butt and Ransom, 1989; Bjartmar et al., 1994).

Figure 40-3 is an electron micrograph showing a maturing oligodendrocyte, together with myelin sheaths that originate from it. Two myelinated axons, the oligodendroglial cell of origin of their myelin sheaths and the thin cytoplasmic processes connecting the oligodendrocyte to the myelin sheaths, are shown. In Figure 40-3 (inset) the thin bridge of oligodendroglial cytoplasm, connecting the myelin sheath to its cell of origin, can be seen at higher magnification.

Figure 40-3 illustrates several important features of oligodendroglial organization, and of the relationship between the oligodendrocyte and myelin sheaths. As noted by Waxman and Sims (1984) and confirmed by Remahl and Hildebrand (1990), the myelin sheaths originating from each oligodendrocyte rotate in random directions, some extending clockwise and the others counterclockwise from their oligodendrocytic stalks. Thus, the myelin circles clockwise around axon A_1 and counterclockwise around axon A_2. The random polarities of the myelin sheaths, despite their origin from the same oligodendrocyte, indicates that the myelin spirals cannot be formed by rotation of the glial cell body around the outer edge of the myelin. On the contrary, the myelin must grow by addition of membrane at, or near, the leading edge of the oligodendroglial processes. This suggests that membrane biosynthesis during myelination is a *local* process, occurring in distal parts of the oligodendroglial process close to the myelin or within the myelin sheath itself.

The myelin sheath is formed by an elaboration of the membrane of the oligodendrocyte, and myelination involves a marked increase in membrane area. The machinery necessary for myelin membrane biosynthesis, in fact, is distributed distally, so that it is located close to the forming myelin sheaths. As shown in Figure 40-3 (inset), polyribosomes and cisternae of ribosome-studded endoplasmic reticulum are present, during development, in distal parts of the oligodendroglial processes, close to the developing myelin sheaths (Waxman and Sims, 1984). A similar proliferation of ribosome-studded endoplasmic reticulum in Schwann cells during myelination in the peripheral nervous system was observed by Robertson (1961), who suggested that this reflects increased membrane biosynthesis during myelination. Transient elaboration of intracytoplasmic membranes, within paranodal oligodendroglial loops associated with maturing myelin sheaths in the central nervous system of developing animals, has also been observed (Waxman et al., 1988). These membranes form tubulovesicular profiles which are most prominent in paranodal loops closest to the node, that is, in parts of the oligodendroglial cytoplasm corresponding to the outermost myelin lamellae, which were probably formed most recently. The presence of these membrane profiles during myelination appears to reflect increased membrane turnover in paranodal oligodendroglial loops, close to the axon. These observations demonstrate a substrate for protein biosynthesis and an intracytoplasmic pool of membrane within distal oligodendroglial processes at sites close to the axon during myelination in the central nervous system. They thus support the hypothesis (Waxman and Sims, 1984) of local membrane assembly within the oligodendroglial processes during myelination.

It appears likely that localized axoglial interactions also participate in the control of myelin sheath characteristics. Myelin formation by a given oligodendrocyte is not stereotyped; it is not, for example, limited to axons of a single diameter. On the con-

trary, single oligodendrocytes can myelinate axons of various diameters in their vicinity. Moreover, a single oligodendrocyte can form myelin sheaths of different thickness around axons of differing diameter. Despite their origin from the same oligodendroglial cell, the myelin sheaths are thicker for larger axons (Waxman and Sims, 1984). The thickness of the myelin sheaths is thus not preprogrammed for each oligodendrocyte but, on the contrary, depends on *local regulation,* by the axon, of myelination.

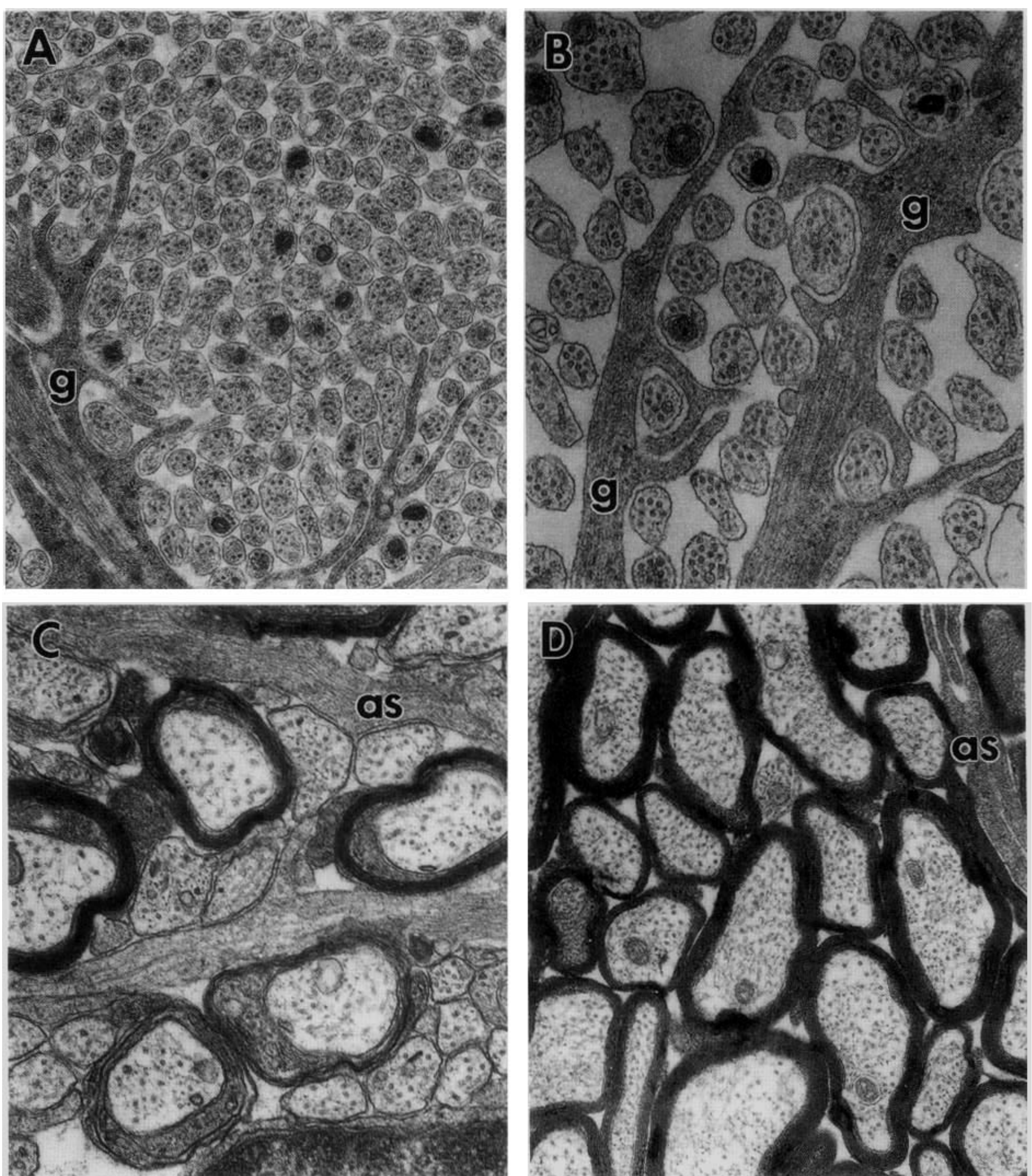

FIG. 40-1. Development of myelinated axons in rat optic nerve, as seen by electron microscopy in transverse section. (A) 2 days postnatal. Occasional septae composed of primitive glial cell processes (*g*) divide the fiber tract into bundles, but most of the premyelinated axons are not yet ensheathed. ×20,000. (B) 8 days old. Occasional axons are loosely ensheathed by primitive, undifferentiated glial cells, but myelin is not yet present. ×40,000. (C) 16 days old. Approximately 20% of the axons are surrounded by compact myelin. ×20,000. (D) Adult. The optic nerve is fully myelinated. ×20,000.

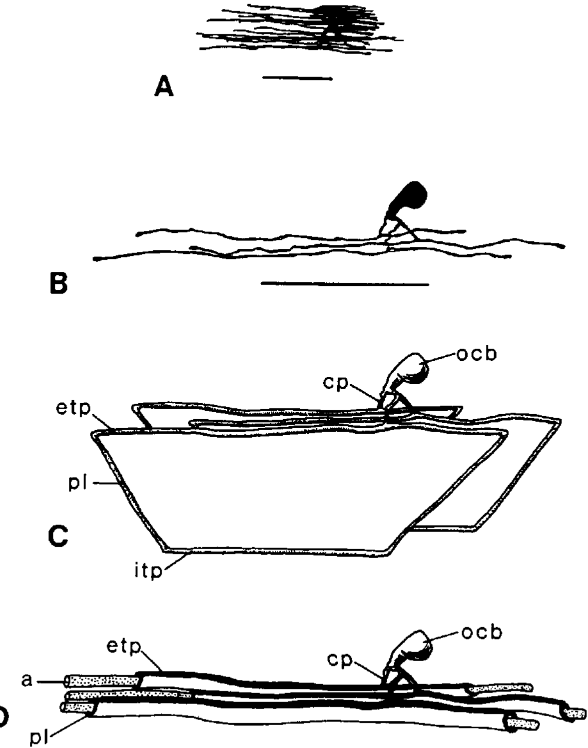

FIG. 40-2. Dye-filled image of a presumed oligodendrocyte. (A) Camera lucida drawing of HRP-filled oligodendrocyte from rat optic nerve (*P12*). Scale bar = 100 μm. (B) Camera lucida drawing of A at higher power (×1250); only the cell body and three selected longitudinal processes have been drawn. Scale bar = 100 μm. (C) Schematic interpretation of the light microscopic morphology of the oligodendrocyte. If the myelin sheaths could be unwrapped they would appear as trapezoid sheets with ridges of cytoplasm along their edges corresponding to the internal and external tongue processes and the paranodal loops; these edges are in cytoplasmic continuity with the cell body. (D) Reconstruction of the oligodendrocyte with its myelin sheaths wrapped around axons. *a,* axon; *ocb,* oligodendrocyte cell body; *cp,* connecting process; *etp,* external tongue process; *itp,* internal tongue process; *pl,* paranodal loop. [Modified from Butt and Ransom (1989), with permission.]

Internode distance along the central nervous system is also matched to fiber diameter (Hess and Young, 1952). The internode distance:diameter ratio is different for fibers in different tracts. Moreover, some central nervous system myelinated fibers exhibit, at specific sites, nonmyelinated zones much larger than normal nodes (Waxman and Bennett, 1972). Thus, it is probable that *localized axoglial interactions* specify the domains of an axon that should, and should not, be myelinated.

Although the molecular mechanisms that control myelination are not yet fully understood, some clues about these mechanisms have been provided by studies of developing central nervous system axons. It is now well established that the axon specifies whether or not a myelin sheath will be formed around it (Aguayo et al., 1976; Weinberg and Spencer, 1976). Freeze-fracture studies have demonstrated a significant increase in intramembranous particle (IMP) density in the protoplasmic face (P-face) of the axon membrane that occurs in association with glial ensheathement in developing white matter tracts (Black et al., 1982). An example is shown in Figure 40-4, which shows a premyelinated axon located adjacent to an ensheathed axon in the developing rat optic nerve. A markedly increased density of P-face IMPs, which correspond to membrane-intercalated protein or glycoprotein molecules, can be seen in the membrane of the ensheathed axon, compared to the bare adjacent axon. The increase in axolemmal P-faced IMP density occurs even in axons that have been experimentally deprived of glial ensheathement (Black and Waxman, 1986), demonstrating that incorporation of new P-face IMPs into the axon membrane does not represent a response to glial contact, but rather reflects an inherent change in axon membrane composition, which occurs at the developmental stage when myelination is usually initiated. The expression of the new axon membrane IMPs occurs only in axons that have achieved the diameter that is associated with myelination, at the time when myelination begins.

Although early hypotheses suggested that myelination was triggered when a developing axon reached a "critical diameter," it is now well established that myelination occurs over a range of diameters (Fraher, 1972), even along single axons (Waxman et al., 1972; Waxman, 1985). The signal for myelination appears to be specific for particular axons or particular domains along axons. The axon membrane contains molecules that trigger mitogenesis in Schwann cells and oligodendrocytes (Salzer et al., 1980a; 1980b; DeVries et al., 1983; Chen and DeVries, 1989). Similarly, the axon membrane appears to express molecules that regulate the rate, and/or degree, of myelin formation. We have hypothesized (Black et al., 1986; Waxman 1987a, 1987b) that incorporation of new axon membrane P-face IMPs reflects the expression of "recognition molecules" in the axon membrane, which initiate myelination. Increased metabolic activity of high molecular weight proteins is observed in Schwann cell membranes following demyelination with lysophosphatidylcholine for several days prior to remyelination (Smith et al., 1983a), consistent with the idea that complementary recognition molecules are synthesized and expressed by myelin-forming cells as myelin production is initiated. The expression in the axon membrane, of a signal that controls myelin formation by nearby glial cells, would provide an explanation for the specific patterns of myelination

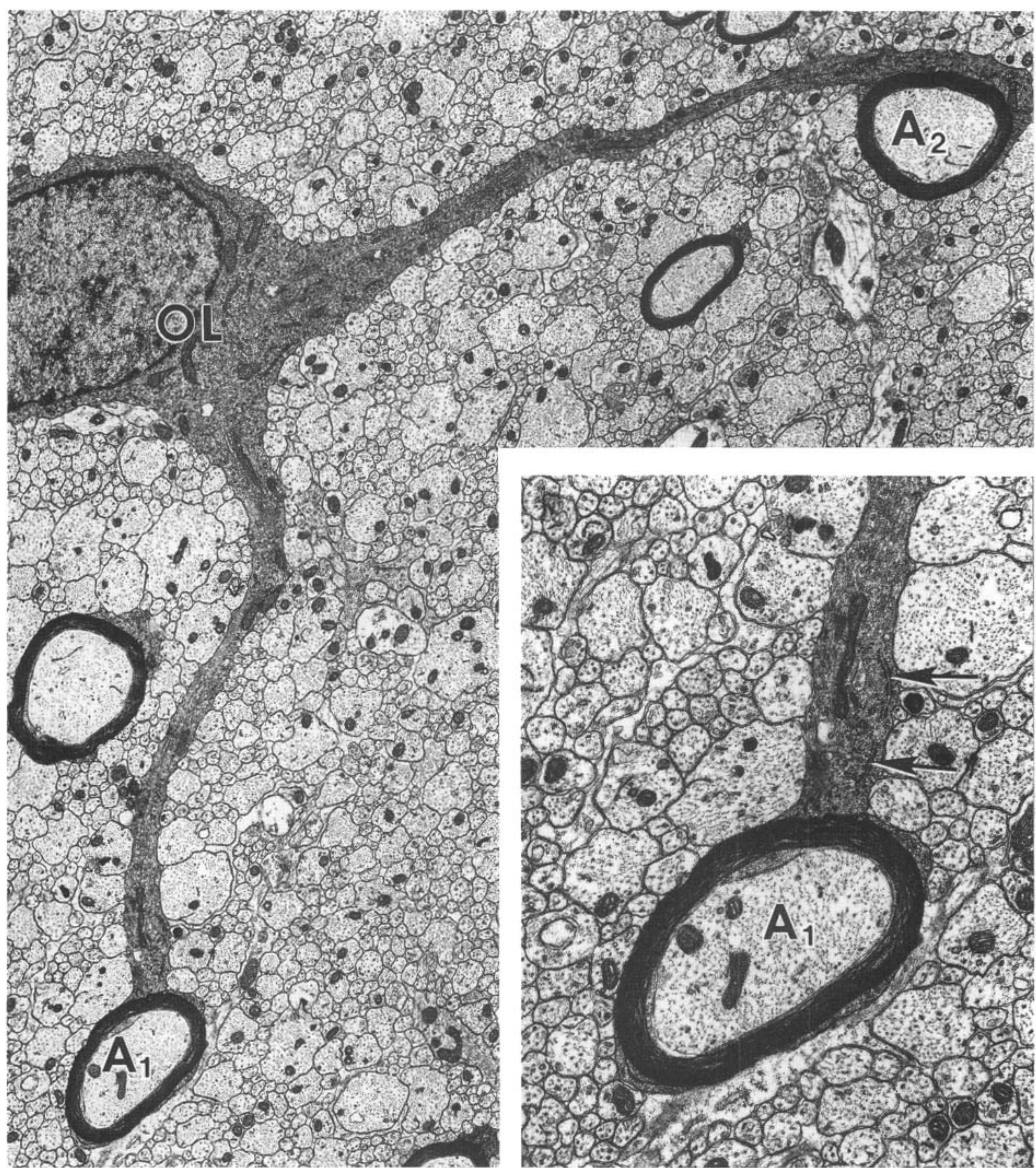

FIG. 40-3. Oligodendrocyte (*OL*) in ventral funiculus of the spinal cord of a 13-day-old rat. Connections can be seen to two myelin sheaths, surrounding axons A_1 and A_2. The myelin sheaths surrounding these two axons have opposite rotational polarities. ×8500. *Inset:* Axon A_1 shown at higher magnification. Note the ribosome-studded endoplasmic reticulum in the oligodendrocyte cytoplasm (arrows) close to the forming myelin sheaths. ×16,000. [Modified from Waxman and Sims (1984), with permission.]

that are observed in the central nervous system (Waxman, 1978). If P-face IMPs, of the type shown in Figure 40-4, represent recognition molecules in the axon membrane that control myelination, the number of axonal recognition molecules per unit axon length would be expected to scale positively with diameter so that the number of axonal recognition molecules presented to a given oligodendrocyte would be greater for axons of larger diameter. According to this scheme, the strength of the axo-

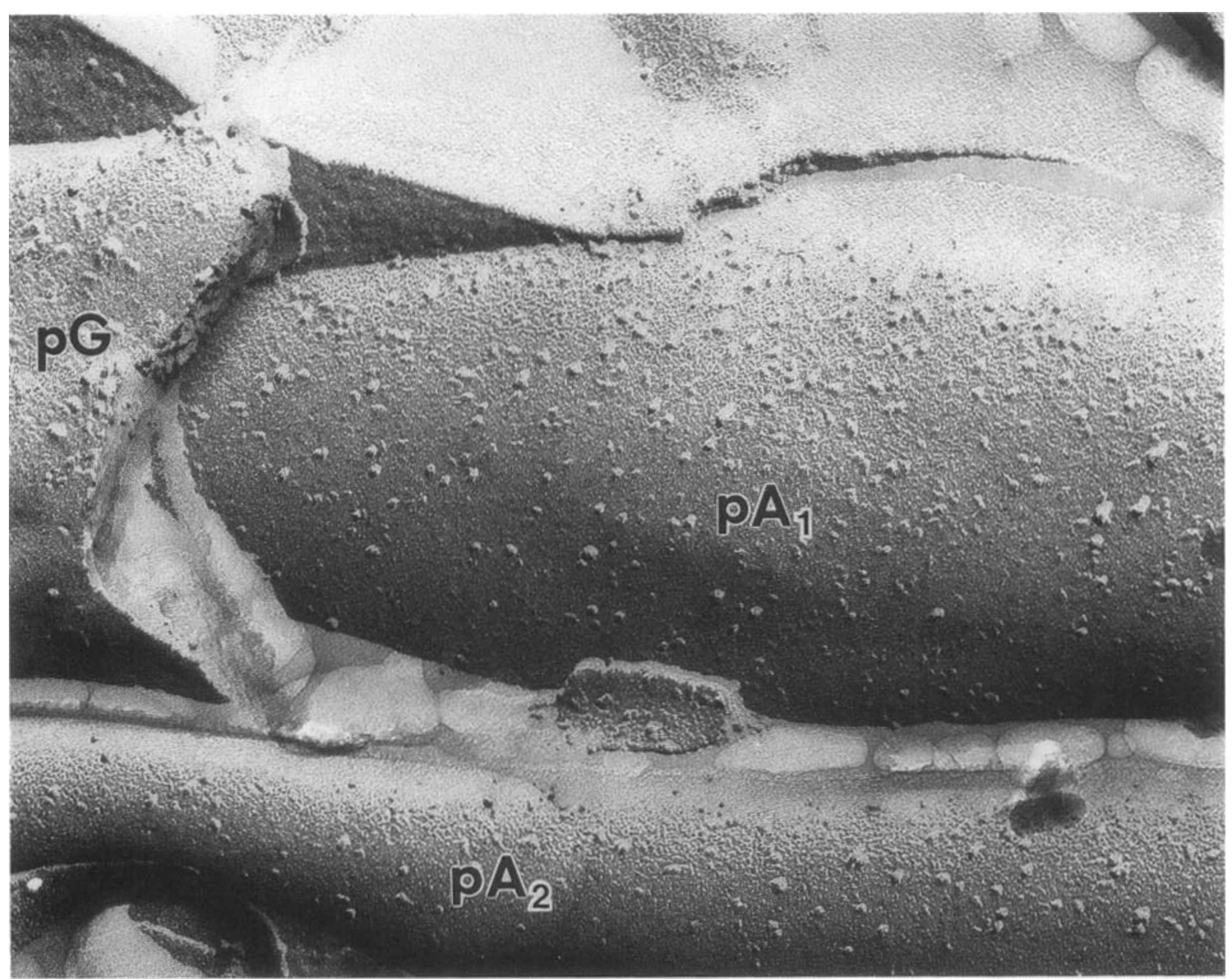

FIG. 40-4. New molecules are incorporated into the axon membrane prior to myelination. This freeze-fracture electron micrograph shows the P-faces of the axon membranes of two adjacent axons from rat optic nerve during the period of myelination. One axon (pA_1) is ensheathed by a glial process whose P-face can be seen (pG). This ensheathed axon expresses a higher density of P-face intramembranous particles than the adjacent premyelinated axon (pA_2) which has not yet elicited ensheathment. ×160,000. [Modified from Black et al. (1982), with permission.]

glial signal would be dependent on diameter, and could thus regulate the thickness of the myelin sheath. The differentiation of the axon membrane might also provide localized signals demarcating single internodes, or larger domains that should or should not be myelinated, since differentiation of the axon membrane into nodal regions (Waxman and Foster, 1980; Waxman et al., 1972) and longer domains destined to remain nonmyelinated (Waxman and Anderson, 1980) occurs prior to the formation of compact myelin.

ASTROCYTE PROCESSES ARE COMPONENTS OF THE NODE OF RANVIER

The early electron microscopic studies of Hildebrand (1971a, 1971b) on feline spinal cord established the presence of perinodal astrocytic processes at nodes of Ranvier in the central nervous system. The ubiquity of perinodal astrocytic processes prompted Hildebrand to suggest that they might participate in essential functions, such as metabolism of the node, the regulation of ion concentrations in the perinodal extracellular microenvironment, or the production of the nodal gap substance. Subsequent studies have demonstrated perinodal astrocyte processes in the rabbit corpus callosum (Waxman and Swadlow, 1976), rat optic nerve (Hildebrand and Waxman, 1984; Waxman and Black, 1984), and guinea pig (Raine, 1984) and rat (Sims et al., 1985) spinal cord. Perinodal astrocyte processes have also been observed at nodes of Ranvier in inframammalian species (Bodega et al., 1987), and the origin of perinodal processes from radial glia has been described (Sims et al., 1991).

Perinodal astrocyte processes usually have sheetlike or fingerlike configurations when reconstructed in three dimensions, and meander or arc through the neuropil for tens of μm, to contact the Na^+ channel-rich membrane at the node of Ranvier. The relationship between the perinodal astrocyte and the neighboring axon is well displayed in freeze-fracture electron micrographs (Figure 40-5), which expose large domains of astrocyte membrane (Waxman and Black, 1984). Perinodal astrocyte processes are closely associated with the axon membrane at the node, approaching within 10 nm of it, but special-

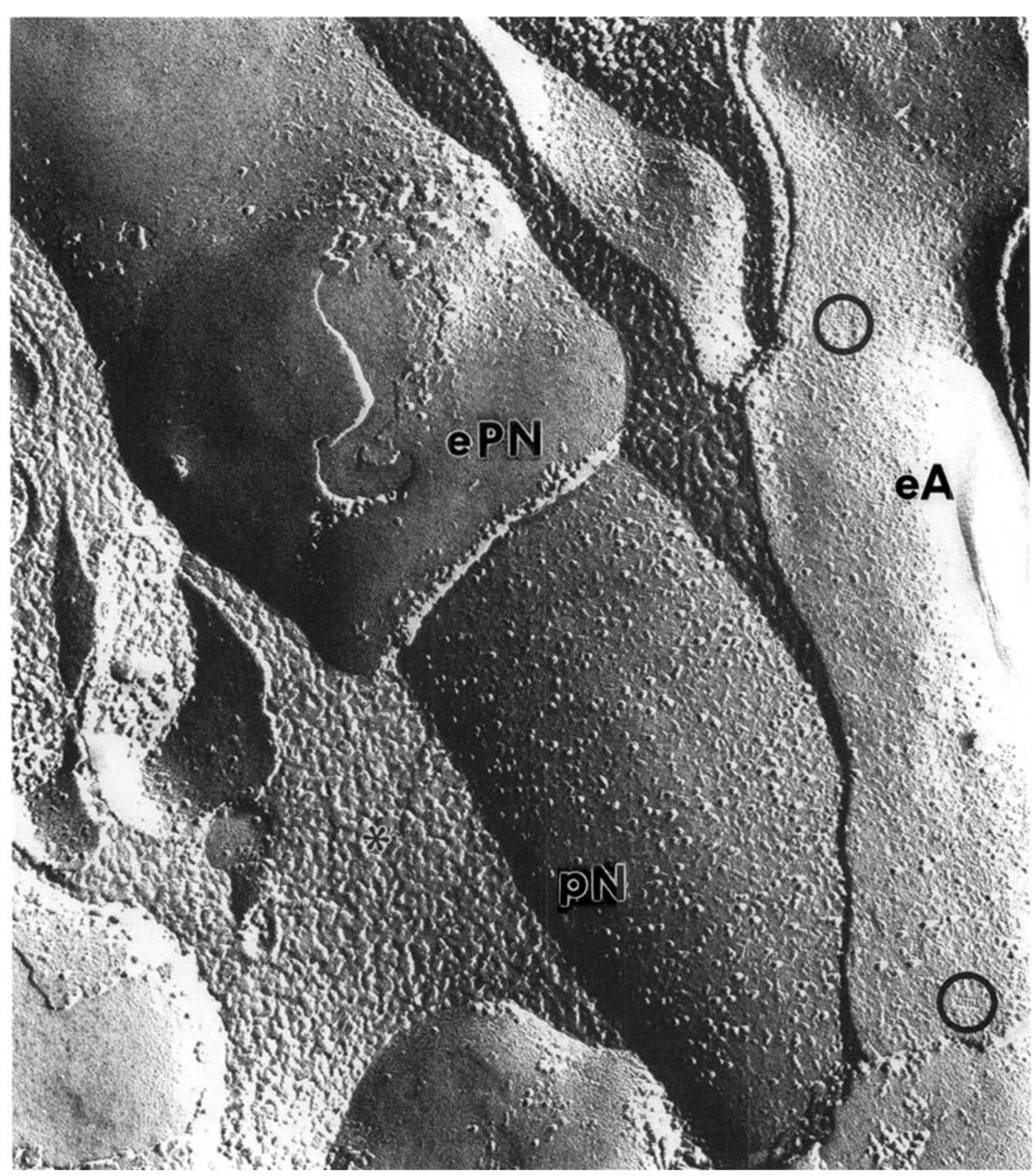

FIG. 40-5. Freeze-fracture electron micrograph showing myelinated axon from rat optic nerve (*pN,* P-face of axon membrane at a node of Ranvier; *ePN,* E-face of paranodal part of oligodendrocyte). A perinodal astrocyte (*eA*), whose E-face is displayed, runs through the neuropil to contact the node. Orthogonal arrays, characteristic of astrocytic membrane, are *circled.* *, extracellular space. ×85,000. [Modified from Waxman and Black (1984), with permission.]

ized membrane junctions, such as gap junctions, tight junctions, and so on, are not present between the perinodal astrocyte and the axon. Gap junctions are present between perinodal astrocytes and the oligodendrocytes bounding the node of Ranvier (Figure 40-6), although the functions of these junctions are not understood (Massa and Mugnaini, 1982; Waxman and Black, 1984).

Astrocyte processes are also associated with Na^+ channel-rich zones of the axon membrane that are not located at nodes of Ranvier. For example, they are associated with patches of node-like membrane that are expressed by specialized nonmyelinated axons such as the nonmyelinated portions of ganglion cell axons within the retinal nerve fiber layer in the rat. These retinal ganglion cell axons are myelinated distally in the optic nerve. Closer to their cell bodies within the retinal nerve fiber layer, these axons are nonmyelinated, but nevertheless express small islands of node-like membrane, which appear to be "hot spots" at which Na^+ channels are clustered (Hildebrand and Waxman, 1983; Black et al., 1984). Radially oriented processes of retinal astrocytes and Müller cells (which are usually considered to be members of the astroglial family) are colocalized with these patches of node-like membrane, and often form a corona around them. The location of the Müller glial processes coincides precisely with that of the node-like axon membrane "hot spots" (Figure 40-7). These nonmyelinated axons thus provide a second example, in addition to the node of Ranvier, of the close relationship between the differentiation of clusters of Na^+ channels in the axon membrane, and abutment of the axon by astroglial processes.

Studies on pathological models have revealed a similar relationship between the astrocyte contact and node-like regions along demyelinated axons. Some of these studies have examined the rat spinal cord irradiated during the period of gliogenesis (at 3 days postnatal) using methods developed by Gilmore (1963, 1966). Since the densities of oligodendrocytes and astrocytes are reduced following irradiation, random neuroglial interactions are minimized, facilitating the study of specific axoglial relationships (Sims et al., 1985). Even though myelin is initially absent in this tissue, small zones of node-

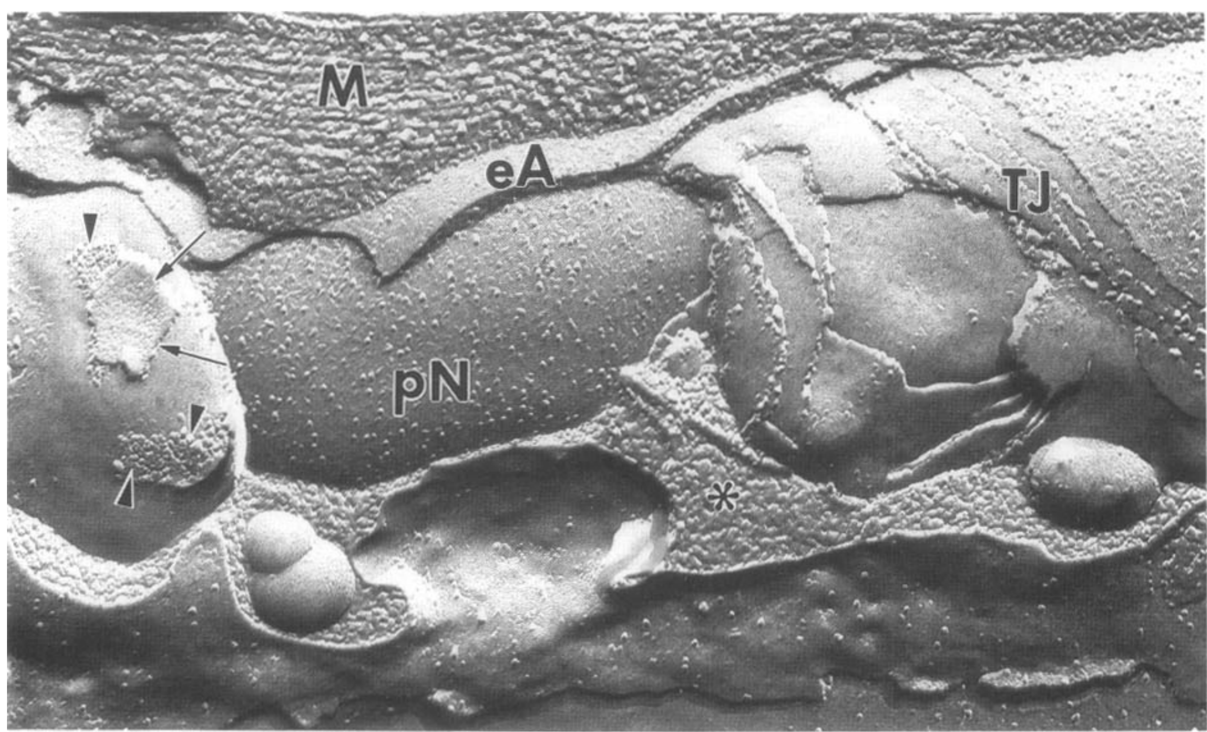

FIG. 40-6. Freeze-fracture electron micrograph showing glial relationships at node of Ranvier from rat optic nerve. The E-face of a perinodal astrocyte process (*eA*) wraps around the P-face of the node (*pN*). Tight junctions (*TJ*) can be seen between oligodendroglial layers in the myelin segment to the right of the node. To the left of the node, the P-face of the outer terminal oligodendrocyte loop is exposed, and two gap junctions are present. The gap junction is formed between the P-face (*arrowheads*) and the E-face (*arrows*) of an adjacent cell. These gap junctions usually connect oligodendrocytes and perinodal astrocytes. *M*, myelin surrounding nearby axon; *, extracellular space. ×58,000. [Modified from Waxman and Black (1984), with permission.]

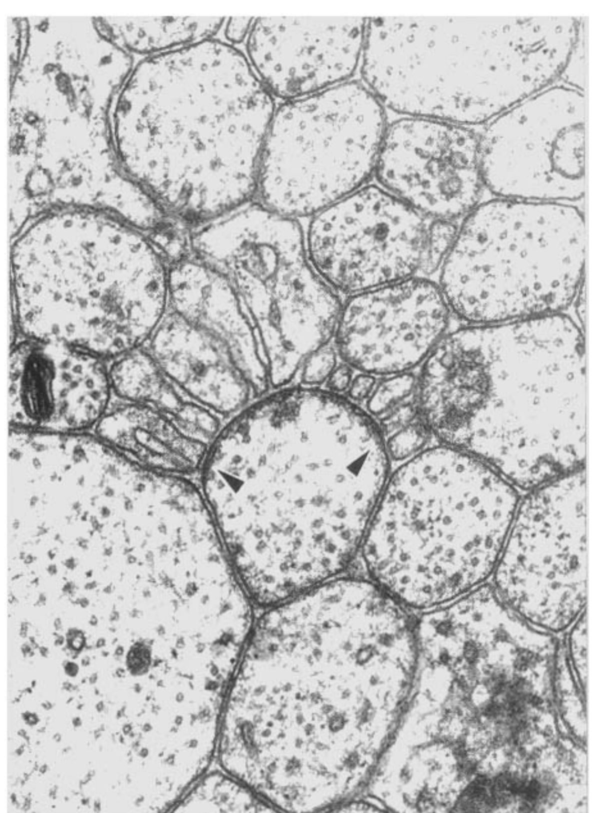

FIG. 40-7. Island of nodelike membrane (*between arrowheads*) in a nonmyelinated axon from retinal nerve fiber layer of adult rat. Note the corona of radially oriented Müller cell processes whose distribution precisely matches that of the nodelike membrane. ×40,000. [Modified from Hildebrand and Waxman (1983), with permission.]

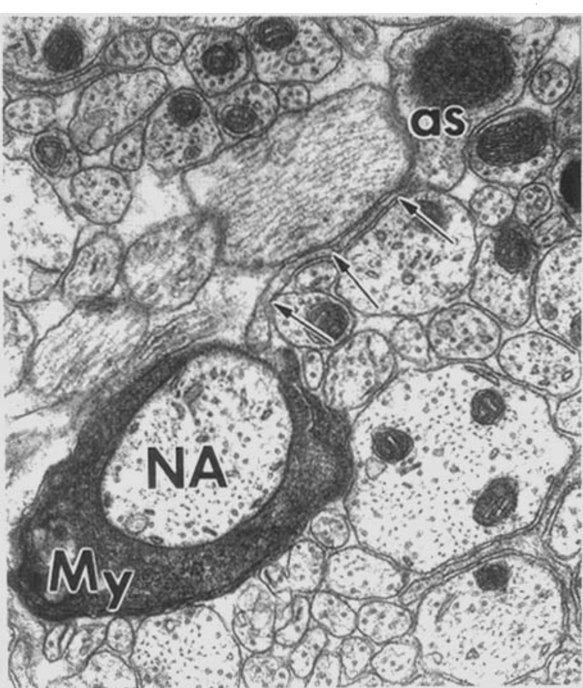

FIG. 40-8. Despite the paucity of glial cells in the dorsal columns of the rat following x-irradiation during gliogenesis, occasional axons are myelinated. This electron micrograph includes a tangential section through a node of Ranvier (*NA*, nodal axon; *My*, myelin). A thin extension (*arrowheads*) runs through the neuropil from an astrocyte process (as) to contact the axon membrane at the node. ×24,000. [Modified from Sims et al. (1985), with permission.]

like membrane develop (at the postnatal age when myelination normally occurs) along some of the unensheathed axons; these zones of node-like membrane are always apposed by astrocyte processes (Black et al., 1985a). Later, following protracted postirradiation intervals, myelination occurs around a small number of axons in the irradiated spinal cord. Despite the paucity of astrocytes in this tissue, astrocyte processes are closely associated with the nodes that do form (Sims et al., 1985). These astrocyte processes can extend for considerable distances through the neuropil to contact the axon at the node (Figure 40-8). The mechanisms that guide the astrocyte processes to their nodal targets and anchor them there are not understood, but clearly there is a high degree of specificity in the astrocyte-axon relationship at the node, both in the normal central nervous system and in glial cell-deprived systems. The Na^+ channel is heavily glyosylated (Trimmer and Agnew, 1989), and it is possible that sialic acid residues, on the extracellular dome of the channel bind, in a manner similar to cell adhesion molecules, to extracellular matrix molecules on the astrocyte surface (Waxman, 1993b). Binding of this type could anchor Na^+ channels at the node, or stabilize the astrocyte-axon contact at nodes of Ranvier.

SODIUM CHANNELS ARE CLUSTERED IN THE NODAL AXON MEMBRANE IN MATURE MYELINATED FIBERS

Voltage-sensitive Na^+ channels are distributed in a highly nonuniform pattern in the axon membrane of mature myelinated fibers. This membrane heterogeneity has been studied using a variety of pharmacological and physiological methods, including saxitoxin (STX) binding (Ritchie et al., 1976), nodal voltage-clamp (Brismar, 1980; Chiu and Ritchie, 1981a; Schwarz et al., 1991), and loose (Shrager, 1987) and gigaseal–patch-clamp methods (Jonas et al., 1989). These methods reveal a high Na^+ channel density (~1,000/μm^2) in the axon membrane at the node of Ranvier (Ritchie and Rogart, 1977; Waxman, 1977; Shrager, 1989) and a much lower density (<25/μm^2) in the internodal axon membrane.

Morphological studies on Na^+ channel distribu-

tion in central nervous system axons have utilized two techniques: freeze-fracture and ultrastructural immunocytochemistry. The freeze-fracture method provides *en face* views of the membrane lipid monolayer closest to the extracellular compartment (E-face), or the lipid monolayer closest to the protoplasm (P-face), and displays intramembranous particles (IMPs), which are thought to represent membrane-associated protein and/or glycoprotein molecules (Branton, 1966; Bullivant, 1977), in the lipid bilayers that partition with either fracture face. The freeze-fracture technique has the advantage of revealing large expanses of the membrane which can be examined *en face*, and it permits quantitative examination of membrane structure with high spatial resolution. Rosenbluth (1976) and Kristol et al. (1977) have presented evidence which suggests that large E-face IMPs are related to voltage-sensitive Na^+ channels. A freeze-fracture electron micrograph showing the E-face of a mature node of Ranvier is shown in Figure 40-9. The nodal part of the axon membrane exhibits a characteristic structure and contains a much higher density of large E-face IMPs than the paranodal and internodal parts of the axon membrane. Even in the absence of definitive identification of the IMPs (if any) that represent Na^+ channels, freeze-fracture permits the visualization of membrane with node-like properties, since the very high density of E-face IMPs makes the nodal membrane morphologically unique.

Immunoelectron microscopic methods, which permit the localization of clusters of Na^+ channels at the electron microscopic level, have recently been developed (Black et al., 1989a, 1989b). These techniques utilize polyclonal antibodies generated against the rat brain Na^+ channel (Elmer et al., 1990) and facilitate the localization of clusters of Na^+ channels. These methods do not, however, permit quantitation of the number of channels (or their density) in a cluster. Moreover, the threshold for staining is not known; it is probably higher than $100/\mu m^2$, since unmyelinated C-fibers, which display Na^+ channel densities of around $200/\mu m^2$ (Pellegrino and Ritchie, 1984), do not display Na^+ channel immunoreactivity with these methods. Figure 40-10 is an electron micrograph, which shows a mature node of Ranvier, immunostained with antibody 7493. This electron micrograph illustrates the dense staining of the axon membrane with antibodies generated against Na^+ channels that is characteristic of the node of Ranvier, in contrast to the internodal and paranodal axon membrane and the oligodendrocyte membrane, which do not stain. The complex structure of the mature node of Ranvier, as shown in Figures 40-9 and 40-10, can be considered the endpoint of development in central nervous system white matter tracts.

THE AXON MEMBRANE HAS A HOMOGENEOUS STRUCTURE PRIOR TO GLIAL ENSHEATHEMENT

The premyelinated axon membrane appears to contain a relatively low density of Na^+ channels, which are distributed uniformly prior to loose wrapping by glial cells. For example, in premyelinated axons of the optic nerve in the neonatal rat, STX-binding experiments demonstrate an Na^+ channel density of approximately $2/\mu m^2$; surprisingly, this low density of Na^+ channels is sufficient to support action potential electrogenesis, probably due to the high input resistance (which is proportional to diameter $^{-3/2}$) in these small-diameter axons (Waxman, et al., 1989). As seen in Figure 40-11, E-face IMP density in these premyelinated axons is quite low and, at early stages of development when glial wrapping is not present, the IMPs are distributed uniformly along the axons and are not clustered (Black et al., 1982). Na^+ channel immunoreactivity is not seen along these immature, bare axons. Thus, prior to glial ensheathement, premyelinated axons do not appear to be regionally specialized. It is likely that Na^+ channels are uniformly distributed along premyelinated axons at early stages prior to glial ensheathement.

As we have discussed above, the mature node of Ranvier is a highly complex structure involving three cell-types and displaying a high degree of axon membrane differentiation. The development of the node, from premyelinated precursor axons, raises four important questions:

1. Does axon membrane differentiation reflect a preprogrammed sequence of events that occurs intrinsically within the neuron, irrespective of association with glial cells?
2. Alternatively, does axon membrane development depend on glioaxonal interactions?
3. How do the oligodendrocyte, astrocyte, and axon collaborate in the differentiation of the mature myelinated fiber?
4. How, and when, do Na^+ channels cluster in the nodal axon membrane, and how does the low internodal Na^+ channel density develop?

We will now examine the complex interactions between the triad of cell types that constitute the central nervous system myelinated fiber—the axon, the oligodendrocyte, and the astrocyte—and will explore the events that regulate axon membrane differentiation during the development of central nervous system white matter.

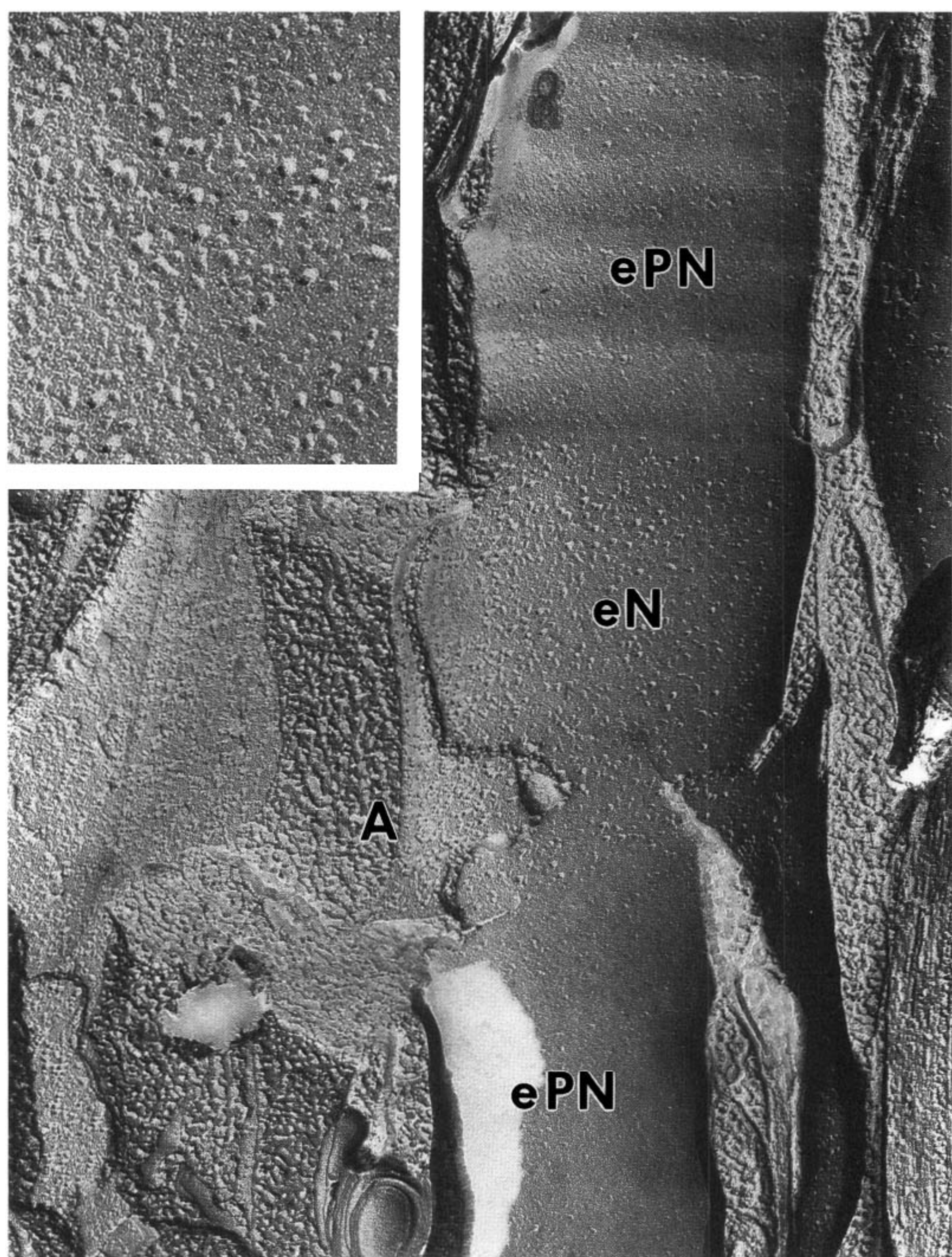

FIG. 40-9. Freeze-fracture electron micrograph showing the differentiated structure of the axon membrane at the node of Ranvier (adult rat, optic nerve-retina junction). The fracture plane exposes the E-face of the axon membrane. Note the difference in the structure of the axon membrane at the node (*eN*), compared with that at the flanking paranodes (*ePN*). A perinodal astrocyte process, which partially encircles the node, is marked *A*. ×70,000. *Inset:* The increased IMP density in the nodal membrane E-face can be more clearly seen at higher magnification. ×175,000.

INSERTION OF SODIUM CHANNELS IN THE DEVELOPING NODAL MEMBRANE IS NOT DEPENDENT ON MYELIN FORMATION

Early studies on developing peripheral nervous system axons used cytochemical and freeze-fracture methods to examine the differentiation of nodal membrane, and demonstrated that the first foci of node-like membrane develop at the time of ensheathment of the axon by loose (noncompact) Schwann cell processes (Waxman and Foster, 1980; Wiley-Livingston and Ellisman, 1980). These node-like

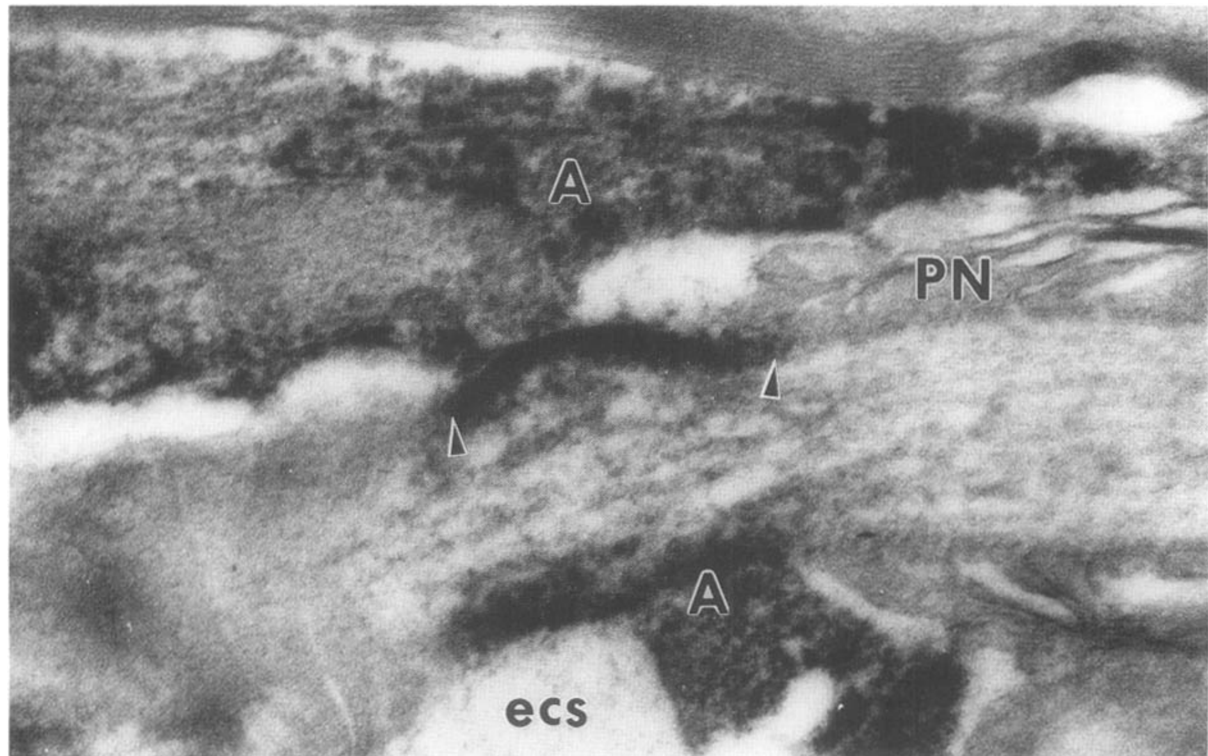

FIG. 40-10. Immunoelectron micrograph showing node of Ranvier from rat optic nerve following staining with antibody 7493 directed against Na^+ channels. There is dense Na^+ channel immunoreactivity of the axon membrane at the node (*between arrowheads*). In contrast, the paranodal axon membrane, beneath the terminating myelin loops (*PN*) is not stained. Dense Na^+ channel immunoreactivity can also be seen within the cytoplasm of an adjacent perinodal astrocyte (*A*). *ecs*, extracellular space. ×60,000. [Modified from Black et al. (1989a), with permission.]

membrane specializations are observed prior to the formation of compact myelin or mature axoglial paranodal junctions, demonstrating that myelination is not a prerequisite for the formation of node-like membrane in peripheral nervous system axons.

A similar sequence of events has been observed in developing central nervous system white matter. Loose glial ensheathement of axons is seen in the rat optic nerve beginning at about 6 days postnatal (Figure 40-1B). The first clusters of large E-face IMPs, similar to those in the nodal axon membrane, appear at the time of loose glial ensheathement. These membrane specializations develop at the edges of ensheathing glial processes (Figure 40-12), and consist of aggregates of IMPs with a higher particle density than found over other parts of the axon membrane. These IMP aggregates contain a high percentage of large IMPs, similar to those seen at nodes of Ranvier. The specialized membrane patches usually extend for 1 to 2 μm along the length of the axon, that is, several times the length of a normal node. The morphology of these IMP clusters suggests that they are primitive Na^+ channel clusters at the precursors of nodes of Ranvier (Black et al., 1982; Waxman et al., 1982).

Glial cell-deficient systems, including the central nervous system of the myelin-deficient *md* rat (Csiza and De LaHunta, 1979; Dentinger et al., 1982) and the dorsal funiculus in the rat spinal cord after x-irradiation (Black et al., 1985a; Sims et al., 1985) have provided additional information about the formation of nodes of Ranvier. In the *md* rat, myelin is absent due to an abnormality in the biosynthesis of proteolipid protein (Duncan et al., 1987; Hudson et al., 1987). To examine the role of myelinating cells in the regulation of Na^+ channel expression, Oaklander et al. (1984) examined the binding of labeled STX, which acts as a marker for Na^+ channels, in 6-day-old and 21-day-old *md* rats, and in normal controls. These ages were chosen because they encompass both the period of active myelination, and the period of development of symptoms in the affected animals. As expected, these workers found a significant, large increase in STX binding capacity of the tissue from 21-day-old normal rats compared to the corresponding tissue from 6-day-old normal animals. However, there was no significant difference in STX binding capacity of *md* and normal brain, spinal cord, or sciatic nerves. Similarly, STX-binding studies on the hypomyelinated murine mutant *shiv-*

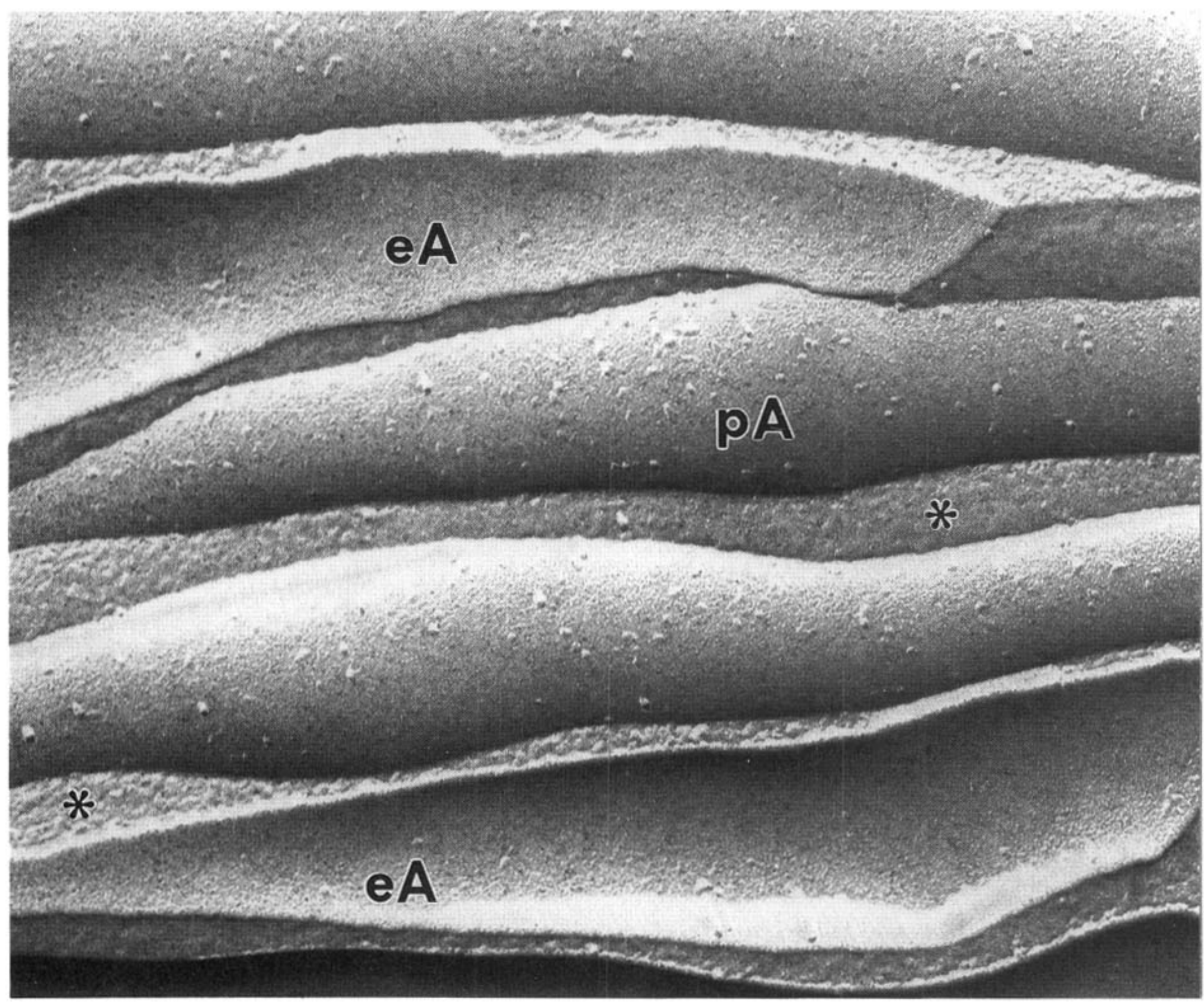

FIG. 40-11. Freeze-fracture ultrastructure of premyelinated axons from 2-day-old rat optic nerve (corresponding to the axons shown in Figure 40-1A). E-faces (*eA*) and P-faces (*pA*) of the premyelinated axons are shown. The E-face intramembranous particle density is low, and regional heterogeneity has not yet developed. ×150,000. [Modified from Black et al. (1982), with permission.]

erer have demonstrated an excess of Na^+ channels compared to control myelinated axons (Noebels et al., 1991). These ligand-binding results do not provide information about the spatial distribution of Na^+ channels; however, they do provide evidence which suggests that, even in the absence of myelination, Na^+ channels are deployed along axons in these hypomyelinated tissues.

Freeze-fracture studies on the x-irradiated spinal cord have provided evidence for the insertion of Na^+ channels in clusters, at sites where nodes of Ranvier would normally develop along white matter axons, irrespective of whether myelination has occurred. Since the number of glial cells is markedly reduced in this model so that most axons are not ensheathed (Gilmore et al., 1982; Sims and Gilmore, 1983), this model permits an examination of axon membrane differentiation in a glial cell-deprived milieu. Black et al. (1985a) observed the development of foci of increased E-face IMP density in spinal cord axons within the irradiated, glial cell-deprived spinal cord at the same ages as in controls, even though axonal ensheathement by myelin-forming cells had not occurred (Figure 40-13). These regions of high E-face particle density extend 1 to 2 μm along the axis of some axons, and up to 5 μm (i.e., about five times the length of a normal node) in others (Black et al., 1985a). These specialized foci contain a high percentage of large (>10 nm) IMPs similar to those at nodes of Ranvier. The total number of IMPs within

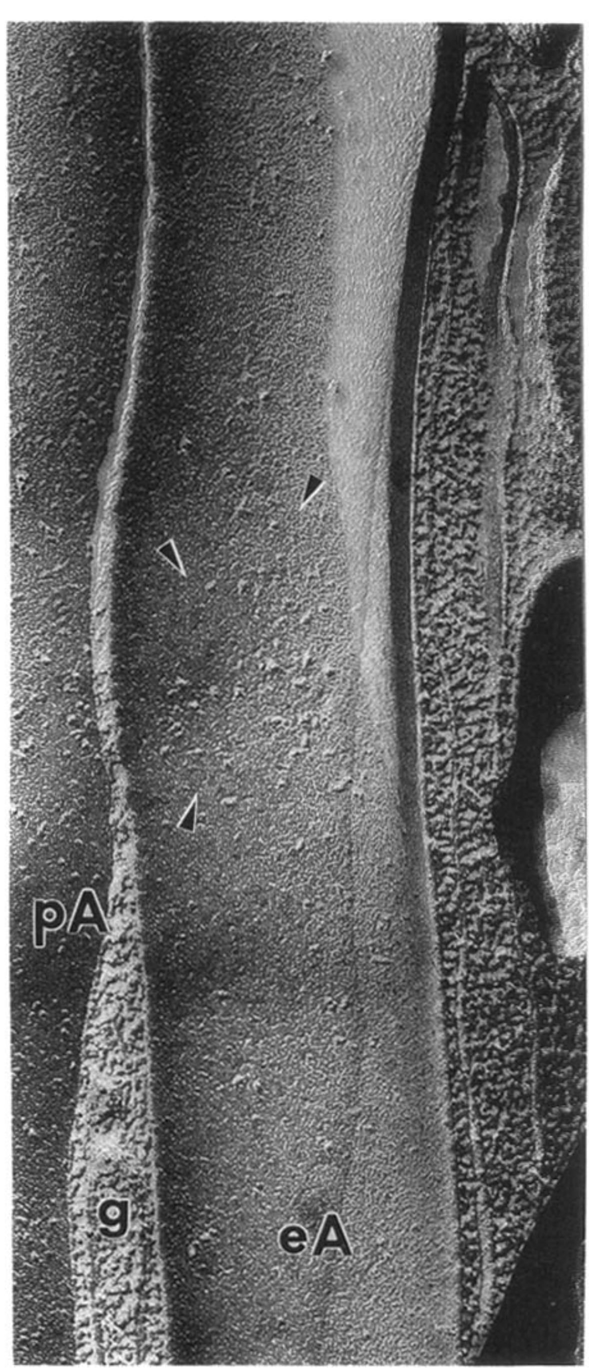

FIG. 40-12. Cluster of E-face intramembranous particles (IMPs) (*between arrowheads*) in the membrane (*eA*) of an axon from 16-day-old rat. This cluster of IMPs probably represents an early concentration of Na^+ channels at a developing node of Ranvier, and is located at the edge of a glial cell process (*g*), which has not yet formed compact myelin. The P-face of an adjacent axon (*pA*) can also be seen. ×100,000. [Modified from Waxman et al. (1982), with permission.]

these regions is similar to that at mature nodes. Thus, even though myelin has failed to develop, foci of node-like membrane are expressed along these axons. Interestingly, these axonal E-face IMP clusters are usually contacted by astrocytes. These foci of axon membrane specialization are likely to be Na^+ channel clusters, and appear to represent the precursors of nodes of Ranvier.

SUBMICRON LOCALIZATION OF NODAL SODIUM CHANNELS IS MODULATED BY INTERACTIONS WITH MYELIN-FORMING CELLS

Although the results described above suggest that Na^+ channels, destined for the nodes of Ranvier, can be inserted into the axon membrane independently of myelination, oligodendrocytes may nevertheless participate in the localization of Na^+ channels in the developing axon membrane. It has been suggested that the formation of a discrete annulus of axon membrane, with high E-face IMP density, may be facilitated by the development of paranodal axoglial junctions between the axon and myelin-forming cells. Rosenbluth (1976) proposed that the paranodal axoglial junctions, between terminating oligodendroglial loops and the axon membrane, might function as barriers that limit the movement of Na^+ channels within the plane of the axon membrane.

Observations on ectopic oligodendroglial processes have provided support for the idea that oligodendrocyte-axon contact modulates the placement of Na^+ channels in the axon membrane (Black et al., 1985b). These observations suggest that at regions where ectopic oligodendroglial processes contact the nodal membrane, there is a focal suppression of Na^+ channels in the axon membrane. Figure 40-14 is a freeze-fracture electron micrograph that shows an aberrant oligodendroglial process, which extends over the axon membrane at a node of Ranvier. The axon membrane has developed a specialized morphology similar to that at the paranodal junctions at the zone of contact between the oligodendrocyte and the axon. In the part of the axon membrane that is contacted by the oligodendrocyte, there is a highly localized reduction in the density of E-face IMPs. The reduction in IMP density is especially significant, since it occurs within the nodal part of the axon membrane where these IMPs are usually expressed with a high density. Since this kind of alteration in axon membrane structure is coextensive with the zone of contact by the oligodendroglial process, this interaction appears to involve a *focal* modulation, by the glial process, of axon membrane

structure. Thus, superimposed upon the inherent differentiation of the axon membrane, contact-mediated interactions with glial cells appear to result in focal alterations in Na^+ channel expression in the axon membrane. These glial-axonal interactions, in the paranodal region, may modulate the structure of the axon membrane during development, so that, at the end of myelination, there is a sharp border that demarcates the axon membrane into nodal and paranodal domains.

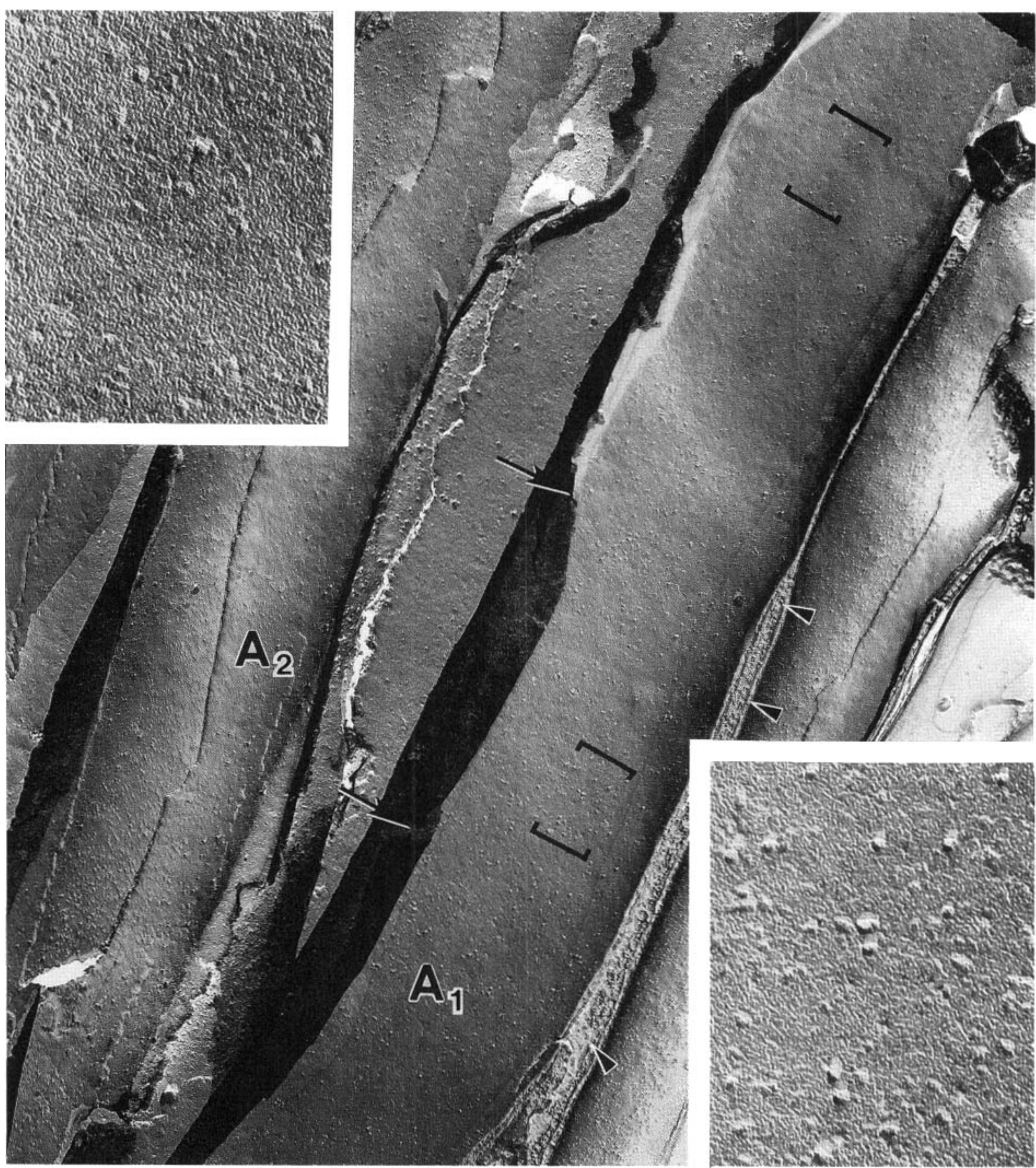

FIG. 40-13. Freeze-fracture electron micrograph showing cluster of E-face intramembranous particles (*between lower brackets*) in an axon (A_1) without myelin in the spinal cord of 19-day-old rat that had been irradiated during gliogenesis. The cluster of E-face intramembranous particles occurs in a region contacted by an astrocyte (*arrowheads*). An adjacent axon (A_2) is not contacted by astrocytes, and does not display clustering of E-face intramembranous particles. ×25,000. *Insets* show the areas in brackets at higher magnification. ×175,000. [Modified from Black et al. (1985a), with permission.]

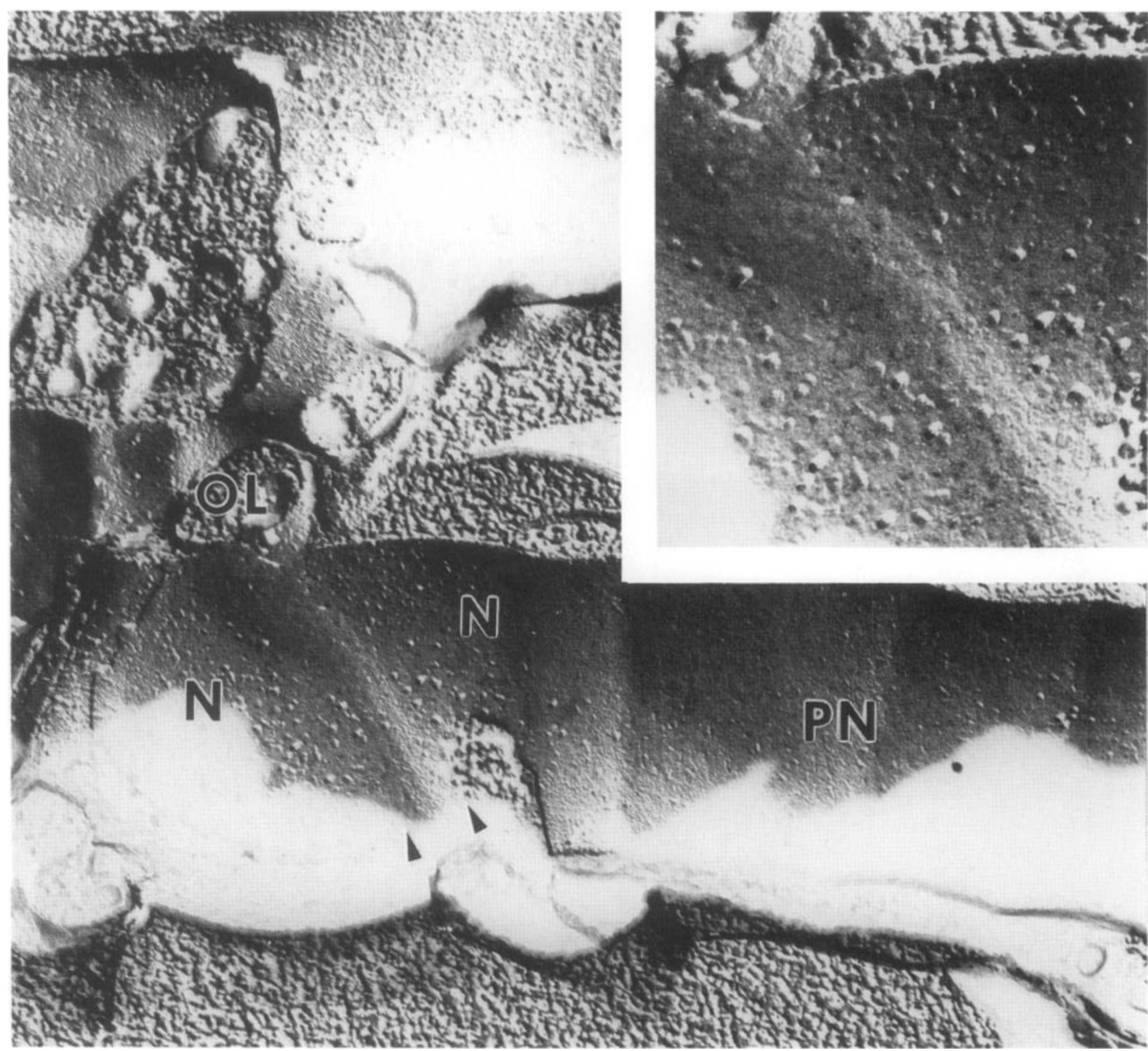

FIG. 40-14. Focal alteration in axon membrane structure at the site of contact with an oligodendrocyte process (*OL*). This freeze-fracture electron micrograph shows the E-face of an axon from the retina-optic nerve junction of rat. Nodal (*N*) and paranodal (*PN*) regions can be seen. An aberrant oligodendroglial process (seen in cross-fracture, *OL*) runs obliquely over the axon membrane (*between arrowheads*). Note the reduction in axon membrane E-face intramembranous particle expression at the zone of oligodendrocyte contact. ×70,000. *Inset:* ×150,000. [Modified from Black et al. (1985b), with permission.]

SUPPRESSION OF INTERNODAL SODIUM CHANNELS FOLLOWS ENSHEATHEMENT BY MYELIN-FORMING CELLS

The development of the axon membrane at the node of Ranvier is described above. A parallel sequence of events occurs as the *internode* develops and becomes myelinated. As described above, in mature myelinated fibers the internodal axon membrane contains a low density of Na^+ channels and may not be excitable (Ritchie and Rogart, 1977; Waxman, 1977; Shrager, 1989). On the other hand, the premyelinated axon (including regions destined to develop into the internodal membrane) is electrically excitable (Foster et al., 1982; Waxman et al., 1989). These observations raise the question of when, and how, the internodal axon membrane becomes inexcitable.

If axon membrane development and myelination were not tightly coupled, developing internodes might become inexcitable prior to myelin formation, so that a transient period of conduction block would occur during development. It might be expected on functional grounds that the internodal axon membrane would maintain its excitability until at least several layers of compact myelin have been

formed, so as to ensure the development of a shield against capacitative current loss and subsequent conduction block. Evidence that loss of excitability in the internodal axon membrane is not a preprogrammed event, but rather involves an active suppression of Na^+ channels by the overlying oligodendrocyte or myelin sheaths, has in fact been provided by studies on glial cell-deprived central nervous system axons (Black et al., 1985a, 1986). E-face IMP density in the unensheathed axon membrane of glial cell-deprived fibers is significantly higher than in age-matched control myelinated (internodal) axon membrane (Figure 40-15). In the dorsal columns of normal rats at 3 days postnatal, the E-face IMP density of premyelinated axons is approximately 300/μm^2. This probably represents a density of Na^+

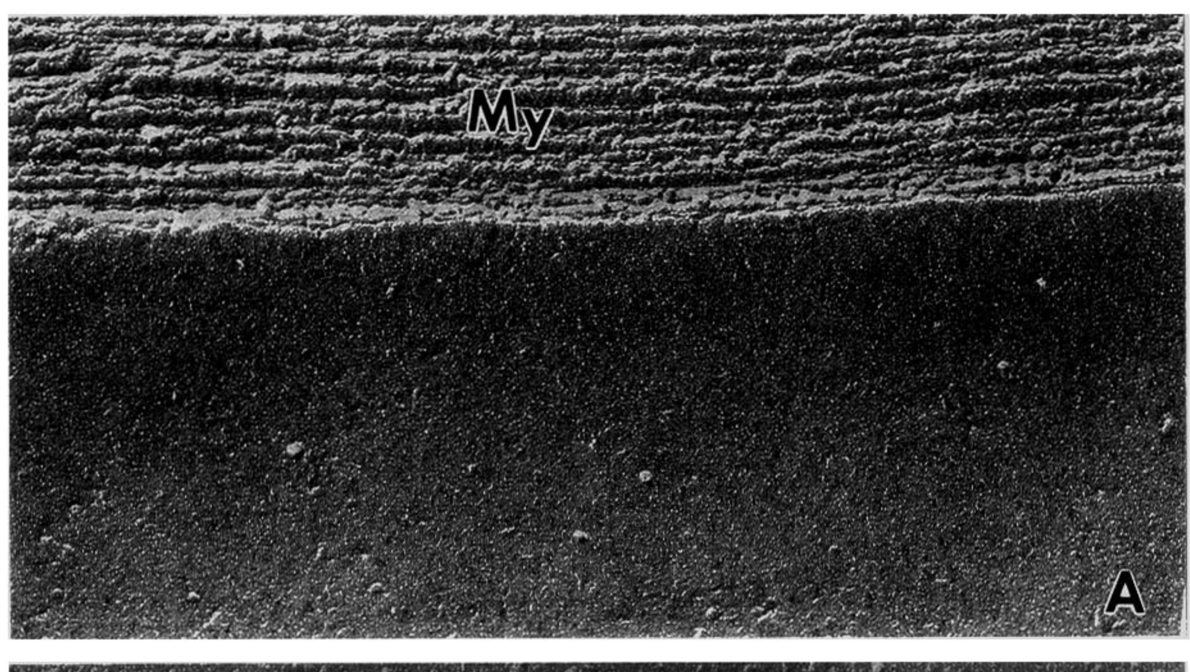

FIG. 40-15. Suppression of E-face intramembranous particles, which may correspond to Na^+ channels, in the internodal axon membrane following myelination. This freeze-fracture electron micrograph shows the axon membrane of myelinated axon (*My*, myelin) from dorsal funiculus of 19-day-old rat (control, *A*), and from glial cell-deprived dorsal funiculus of rat that had been irradiated during gliogenesis (*B*). In the absence of glial ensheathment, E-face IMP density remains high, consistent with a relatively high Na^+ channel density. ×150,000.

channels similar to that in nonmyelinated axons. During normal development, the density of E-face IMPs in the axon membrane is reduced as the axon is myelinated, falling to approximately 180/μm^2 in the internodal axon membrane in 19-day-old myelinated axons. The reduction in E-face IMPs appears to be related to the development of inexcitability of the internodal part of the axon.

Formation of myelin around the axon appears to be a prerequisite for the reduction in Na^+ channel expression in the internodal axon membrane; if myelination is delayed, the loss of Na^+ channels does not occur. Thus, in unensheathed axons in the spinal cord of the 19-day-old x-irradiated rat (which, on the basis of diameter, would normally have been myelinated), there is not a reduction in the E-face IMP density (Figure 40-15B). On the contrary, E-face IMP density in these glial cell-deprived axons increases slightly, to approximately 440/μm^2. This value is similar to that observed in unmyelinated fibers in the 19-day control spinal cord (approximately 450/μm^2), and it suggests that a density of Na^+ channels similar to that in unmyelinated axons (and therefore sufficient to support conduction) is retained when myelination is prevented (Black et al., 1985a). Behavioral observations provide evidence for maintenance of conduction in the glial cell-deprived axons since paraplegia is not observed in developing rats after x-irradiation.

The maintenance of Na^+ channel densities, at levels higher than in the internode, in glial cell-deprived axons raises the important question: What are the mechanisms that downregulate Na^+ channel density in the axon membrane after myelination? One possibility is that myelination provides a signal that results in a suppression of Na^+ channel expression. There is precedent for downregulation of Na^+ channel expression via cell-cell interactions; Na^+ channel expression in spinal cord astrocytes is decreased when these cells are cocultured together with dorsal root ganglion cells (Thio et al., 1993). If excitability is lost in the internodal axon via suppression of Na^+ channels as a result of myelination, it would be predicted that, even if myelination occurred in a delayed manner, it would be accompanied by a decrease in axon membrane E-face IMP density. In the irradiated spinal cord, some axons are, in fact, myelinated after a delay of several weeks by either oligodendrocytes or Schwann cells. When spinal cord axons are examined after delayed myelination, they display a significant reduction in E-face IMP density, which falls to approximately 150/μm^2 (a value similar to that of normal internodal membrane) in the newly formed internodal axon membrane (Black et al., 1986). The reduction in E-face IMP density is seen with both oligodendrocyte- and Schwann cell-mediated myelination. Since, in neighboring axons that had not been myelinated, E-face IMP densities are maintained at high levels (approximately 400/μm^2) the change in axon membrane structure is related to myelination, and is not a generalized systemic change (Black et al., 1986).

On the basis of these observations, we have proposed that normal axonal maturation involves a *suppression of internodal* Na^+ *channel expression,* which occurs as a result of ensheathment by myelin-forming cells (Waxman, 1987a, 1987b). The mechanism underlying the suppression of Na^+ channels is unclear. Since Na^+ channels are heavily glyosylated (Trimmer and Agnew, 1989), cell-cell interactions involving binding between glial cell-associated molecules and Na^+ channels could modulate their channel expression or localization (Waxman, 1993b). Alternatively, the decreased transmembrane voltage gradient due to the voltage drop through the overlying myelin could affect channel turnover and result in a reduced Na^+ channel density in the internodal axon membrane. Although the half-life of Na^+ channels in myelinated axons is not known (and may be different for premyelinated, nodal, and internodal Na^+ channels), the half-life of Na^+ channels in neuroblastoma cells and Schwann cells is 26 hours and 2.2 days, respectively (Waechter et al., 1983; Ritchie, 1988). If myelination were to be followed by a structural alteration which reduced the rate of insertion of Na^+ channels in the underlying axon membrane, there would be a decrease in the density of Na^+ channels in the internodal membrane over the next several days due to channel turnover and lack of replenishment.

ASTROCYTES ARE INVOLVED IN THE CLUSTERING OF SODIUM CHANNELS IN THE AXON MEMBRANE

Following early patch-clamp studies that demonstrated Na^+ channels in cultured astrocytes (Bevan et al., 1985; Gray and Ritchie, 1985), it has been shown in a number of studies that mammalian astrocytes *in vitro* can express Na^+ channels (Nowak et al., 1987; Barres et al., 1988; Sontheimer et al., 1991a, 1991b, 1992; Sontheimer and Waxman, 1992). Moreover, some mammalian astrocytes can express Na^+ channels with biophysical properties similar to Na^+ channels expressed by neurons (Barres et al., 1989; Sontheimer and Waxman 1992; Sontheimer et al., 1991a, 1991b). Using *in situ* hybridization, Black et al. (1994a) detected the expression of mRNA for Na^+

channel subtypes II and III, as well as the putative glial cell-specific Na^+ channel NaG (Gautron et al., 1992) in cultured astrocytes. The possibility that astrocytic Na^+ channel expression is an artifact of culture was ruled out by immunoultrastructural studies which used polyclonal antibodies directed against Na^+ channels (Black et al., 1994b) to demonstrate that Na^+ channels are present within perinodal astrocytes *in situ* within the mammalian central nervous system. In addition, RT-PCR (reverse transcriptase polymerase chain reaction) amplifies the mRNAs for Na^+ channel α-subunit subtypes I, II, and III from rat optic nerve, which does not contain neuronal cell bodies but does contain astrocytes (Oh et al., 1994), and also amplifies the Na^+ channel $\beta1$ subunit (Oh and Waxman 1994; Oh et al., 1994).

The distribution of Na^+ channels within astrocytes in the optic nerve is nonuniform; immunoreactivity with antibodies generated against Na^+ channels is more intense in perinodal astrocyte processes, close to nodes of Ranvier, than in astrocyte cell bodies or endfeet surrounding blood vessels or near the glia limitans (Black et al., 1989b). The subcellular localization of Na^+ channels is different, moreover, in astrocytes and axons. Within myelinated axons, immunoreactivity with antibodies generated against Na^+ channels is most intense in the axon membrane at the node of Ranvier, and there is only moderate immunoreactivity within the axoplasm. In contrast, in astrocytes the Na^+ channel immunoreactivity is distributed throughout the cytoplasm (Black et al., 1989a, 1989b). These results suggest that, within astrocytes, there is a cytoplasmic pool of Na^+ channels or channel precursors. Although STX-binding studies have not yet been carried out on astrocytes to determine the magnitude of the cytoplasmic pool of Na^+ channels, STX-binding studies have been carried out on Schwann cells (which also exhibit Na^+ immunoreactivity within the cytoplasm). These binding studies have demonstrated that approximately 50% of saturable STX-binding sites are located within the cytoplasmic compartment of Schwann cells (Ritchie et al., 1990). Differences in the equilibrium dissociation constant K_D between the cytoplasmic and membrane-associated pools of Na^+ channels suggest a precursor-product relationship, as has been observed in axonal growth cones (Wood et al., 1989). This is consistent with the idea that the cytoplasmic pool contains Na^+ channel precursors (Ritchie et al., 1990). The presence of a similar intracytoplasmic pool of Na^+ channels in astrocytes *in situ* is suggested by the observation of immunoreactivity with antibodies generated against Na^+ channels within the cytoplasm of astrocytes in the optic nerve (Black et al., 1989a, 1989b).

While Na^+ channels are present within the membranes of most astrocytes that have been studied, they are apparently distributed with a density ($<1/\mu m^2$) (Sontheimer et al., 1991a, 1991b) that is too low to support electrogenesis [an exception is observed in spinal cord cultures, where some astrocytes can transiently express high Na^+ channel densities, up to 8 to $10/\mu m^2$ during the second week *in vitro*, although action potential generation does not occur, since these cells have a low resting potential so that most channels are inactivated at rest (Sontheimer and Waxman, 1992; Sontheimer et al., 1992)]. Moreover, astrocytes typically have significantly greater K^+ conductances than Na^+ conductances; the large P_K:P_{Na} ratio stabilizes membrane potential close to E_K and thus inhibits excitability (Sontheimer et al., 1992). In addition, electrical coupling between astrocytes (Gutnick et al., 1981) will tend to clamp each cell close to a common value for the population, impeding the rapid regenerative responses necessary for action potential electrogenesis.

The possibility that astrocytes might function as subsidiary sites for the synthesis of Na^+ channels which are transferred to the axon membrane has been suggested by Ritchie and his colleagues (Bevan et al., 1985; Gray and Ritchie, 1985). Cell-to-cell transfer of ion channels has not been demonstrated to date, and this hypothesis thus remains speculative. Nevertheless, the available evidence suggests that glial cells participate in the development of Na^+ channel clusters as the axon develops. The earliest foci of node-like membrane develop along premyelinated axons at a time when they have become associated with glial cells (Black et al., 1982; Waxman et al., 1982; Tao-Cheng and Rosenbluth, 1983). Moreover, preliminary observations suggest that, even at early stages in the development of Na^+ channel clusters along premyelinated axons (i.e., at early stages in the development of nodes of Ranvier), the axon is associated with glial cells that contain Na^+ channels. Figure 40-16 is an electron micrograph, showing a longitudinal section through the optic nerve of a 10-day-old rat. The tissue has been immunostained with an antibody directed against the rat brain Na^+ channel. Although compact myelin has not yet been formed, one axon expresses a small zone of Na^+ channel immunoreactivity, which extends approximately 1.5 μm along the length of the fiber, a distance that is slightly longer than the length of a mature node of Ranvier. This "hot-spot" of Na^+ channel immunoreactivity appears to be the precursor of a node of Ranvier (see Waxman et al., 1982). Flanking this part of the axon, two cellular processes, containing dense Na^+ channel immunoreactivity, are present. The location and orientation of

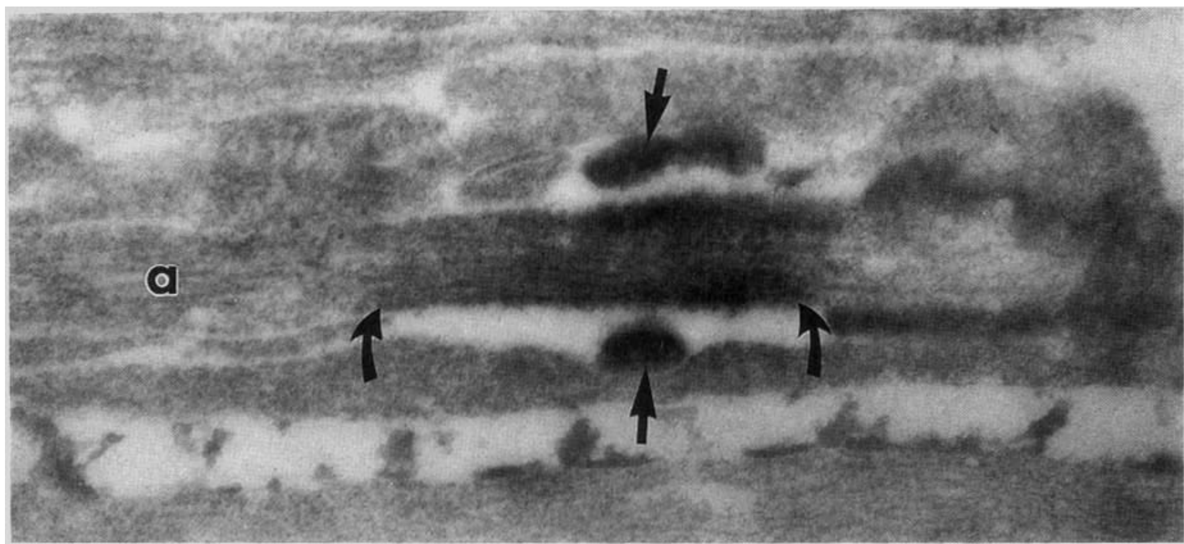

FIG. 40-16. Perinodal astrocytes appear to be present even at early stages of nodal development. This electron micrograph shows, in longitudinal section, the premyelinated optic nerve from a 10-day-old rat, immunostained with antibody 7493. An island of dense Na^+ channel immunoreactivity (*between curved arrows*) is present along a premyelinated axon, at a region where a node of Ranvier will presumably develop. This hot spot of axonal Na^+ channel immunoreactivity is flanked by two glial processes (*straight arrows*), which also express Na^+ channel immunoreactivity. The orientation and size of these glial processes, as well as their location, are similar to those of perinodal astrocytes. ×40,000.

these processes are similar to that of perinodal astrocytes. While, in the absence of double staining for GFAP (glial fibrillary acidic protein) or another cellular marker for astrocytes, it is impossible to definitively identify these processes as astrocytic, these preliminary results suggest that, even at early stages of maturation, astrocytes containing Na^+ channels are associated with developing nodes of Ranvier.

ASTROCYTES ARE INVOLVED IN AXONAL REORGANIZATION AFTER DEMYELINATION

Astrocyte processes, and their Schwann cell counterparts in the peripheral nervous system, may also play a role in Na^+ channel reorganization along demyelinated axons. Smith et al. (1983b) studied demyelinated axons in peripheral nerve following injection of lysophosphatidylcholine, and observed that, prior to remyelination, discontinuous conduction was supported by "phi-nodes," which presumably correspond to clusters of Na^+ channels. In an electron microscopic study, Blakemore and Smith (1983) observed a possible morphological counterpart for phi-nodes, that is, islands of axon membrane that were associated with a dense undercoating, similar to the nodes of Ranvier. These specialized regions of the axon membrane were always contacted by Schwann cell processes. Within the spinal cord, chronically demyelinated axons also exhibit zones of node-like specialization. These are invariably abutted by astrocyte processes (Rosenbluth and Blakemore, 1984; Black et al., 1991). In an immunoultrastructural study on Na^+ channel localization along dorsal column axons following ethidium bromide irradiation in the rat, Black et al. (1991) demonstrated that, by 4 weeks following demyelination, immunoreactivity with antibodies generated against Na^+ channels develops along some demyelinated spinal cord axons. The Na^+ channel-rich membrane is only seen in axons following contact by glial cells.

There is a remarkable degree of overlap between the region of Na^+ channel clustering and the zone of glial contact along demyelinated axons (Black et al., 1991). Some chronically demyelinated axons express discrete "islands" where Na^+ channels are clustered in high density. Whenever such clusters of Na^+ channels are observed, they are abutted by glial cell processes whose extent very closely matches the distribution of the Na^+ channel-rich axon membrane (Figure 40-17). Precise overlap, between astrocyte-axon contact and development of nodelike properties in the axon membrane following demyelination, suggests that astrocyte-axon interactions play a role in reorganization of the axon membrane following demyelination. Thus, during both normal development (see Figure 40-16) and following demyelination (Figure 40-17), there appears to be a close relationship between astrocyte-axon contact and increased expression of Na^+ channels in the axon membrane.

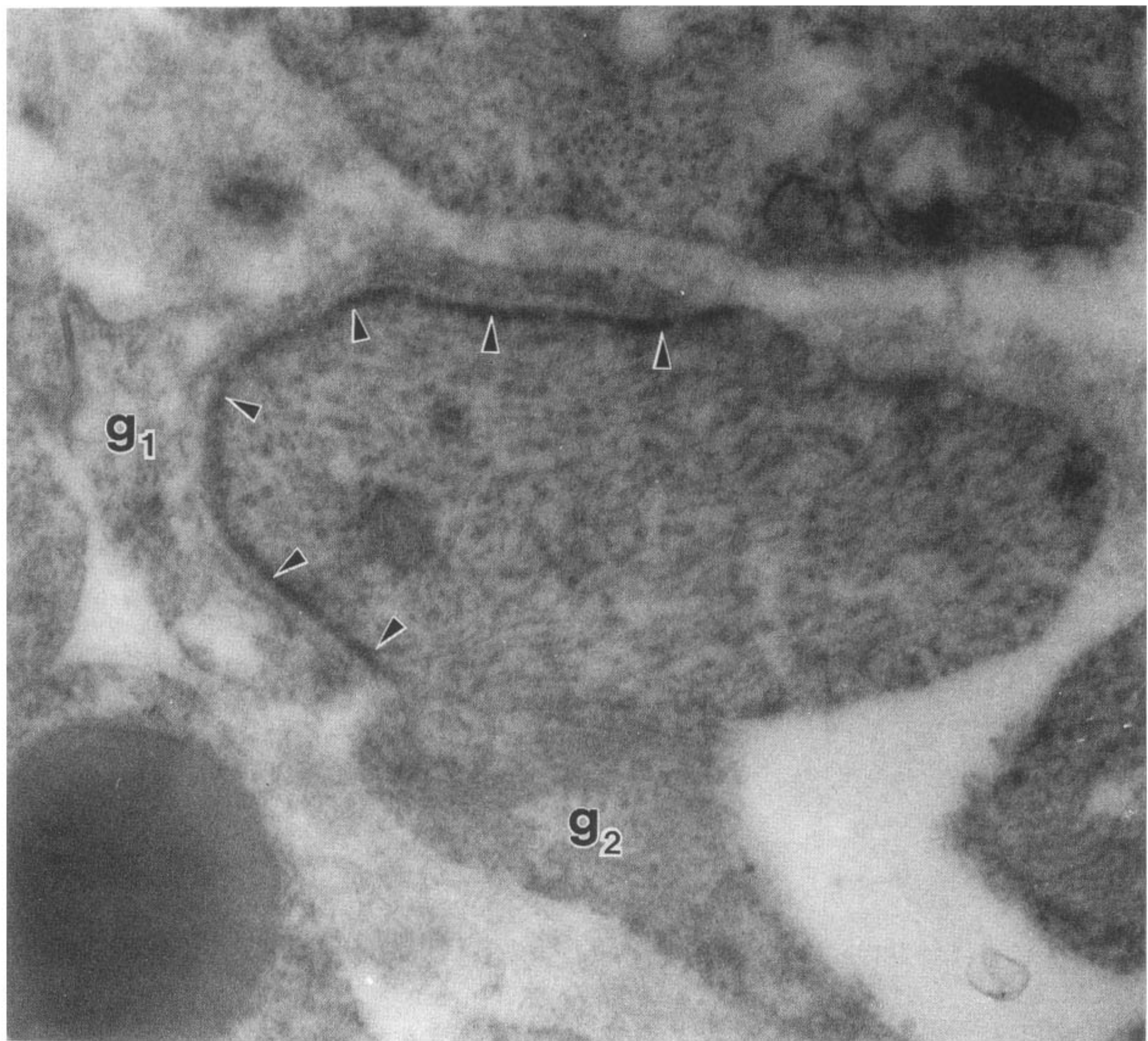

FIG. 40-17. Glial processes are present at hot spots of Na^+ channels along demyelinated axons. This immunoelectron micrograph shows Na^+ channel expression in axons in the spinal cord (dorsal column) of a rat that had been demyelinated with ethidium bromide 30 days previously. An island of Na^+ channel immunoreactivity (*arrowheads*) is present in part of the axon membrane, and is abutted by a glial process (g_1). The axon membrane contacted by a second glial process (g_2) does not express Na^+ channel immunoreactivity. ×50,000. [Modified from Black et al. (1991), with permission.]

Gliosis has classically been considered as a *negative* response to injury, that is, a response that inhibits recovery of function. Classical pathologists, for example, proposed that astrocytes inhibit central nervous system regeneration and remyelination. It is now clear that astrocytes also exhibit a number of *positive* roles, which may promote recovery of function, following injury to the brain and spinal cord. This conclusion has important implications for a number of disorders, including multiple sclerosis and spinal cord injury. For example, under some circumstances astrocytes may promote the regrowth of central nervous system axons (Liesi and Silver, 1988; Schwab and Caroni, 1988). Similarly, as discussed above, astrocytes may participate in the reorganization of Na^+ channels in demyelinated axons (Waxman, 1993a). The astrocyte is clearly emerging as a complex cell, which is intimately related to the axon, both in the normal central nervous system and following various pathological insults. It may be simplistic to think that functional recovery can be promoted simply by muting the astrocytic response to injury. In fact, the complexity of the astrocyte's role, both in normal development and following central nervous system injury, suggests that it might be therapeutically advantageous to enhance some aspects of the astrocytic response to injury of the brain and spinal cord.

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VIII Membrane Structure and Cytoskeletal Proteins

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41 Glial membranes and axoglial junctions

JACK ROSENBLUTH

This chapter is concerned primarily with the plasma membranes of macroglia and their interactions at specialized interglial and axoglial contacts. The functional significance of specific surface structures and junctions are considered. Most of the discussion will be devoted to myelin-forming cells; astrocytes are reviewed extensively elsewhere (Chapters 19, 27, 28, and 40).

SIZE AND SHAPE OF MYELIN-FORMING CELLS

Classically, myelin-forming glial cells are depicted without their attached myelin sheaths, and the misleading impression has thus been fostered by neurohistological studies that oligodendrocytes, in particular, are small cells that give rise to few processes. In the light of more recent work, including dye injection studies (Butt and Ransom, 1989), however, it is clear that these cells, far from being small, are among the largest known by virtue of their gigantic membranous appendages, the myelin sheaths, which presumably remain attached to the cell body under normal circumstances. Myelin-forming cells rival neurons with respect to surface area, which is orders of magnitude greater than that of typical epithelial cells having perikarya of comparable size. This enormous surface is divided into distinct domains with regard to the distribution of both cytoplasmic and plasma membrane components.

A Schwann cell produces a single myelin sheath around one axonal segment ~100 to 1000 μm in length. Oligodendrocytes, in contrast, may generate multiple myelin segments around different axons. However, the size of the fibers and the thickness of the sheaths are very different in the peripheral and central nervous systems, and, as a result, the overall surface area of myelin generated by an oligodendrocyte around multiple axons may be no larger than that formed by a Schwann cell around a single internode.

For example, the total surface area of a myelin-forming Schwann cell, based on an internodal length of 500 μm, an axon diameter of 8 μm, and 50 myelin lamellae, would approximate 0.6 mm^2, ~96% of it in the domain of the compact myelin lamellae. An oligodendrocyte that forms myelin around 10 axons, each 2 μm in diameter with an internodal length of 500 μm and 20 layers in each sheath, would also have a total surface area of ~0.6 mm^2. In contrast, a cuboidal epithelial cell 10 μm wide would have a total surface area of <0.005 mm^2, that is, more than two orders of magnitude less.

A neuron with an axon 2 μm in diameter and 10 cm long would have an overall surface membrane area approximating 0.6 mm^2 as well. However, such a neuron would have a correspondingly large cytoplasmic volume to support, while the myelin-forming cell would not; the glial plasma membrane is thus disproportionately large, and the cell body supporting it is presumably involved primarily in the maintenance of that membrane.

The cell surface area of myelin-forming cells is so large as to suggest the need for specialized structures and mechanisms for transporting components between the perikaryon and the remote extensions. Microtubules, which are conspicuous in the cytoplasm-containing portions of myelin-forming cells, may subserve the directional movement of organelles within glial cells, as they do in axons. Post-Golgi vesicles transported in this way could be involved in the growth, turnover, or modification of compact myelin at distant sites.

Autoradiographic studies of turnover do indeed demonstrate involvement of the superficial cytoplasmic channels of Schwann cells in the synthesis and distribution of myelin components to the sheath. Choline lipids spread rapidly inward through the compact myelin, while proteins and glycoproteins tend to remain more superficial. The role of the paranodal loops and the Schmidt-Lanterman clefts is uncertain. They could be involved in signaling from the axon to the perinuclear portion of the Schwann cell (Gould, 1990) as well as in the transport of cytoplasmic organelles from the Schwann cell perikaryon to its periphery.

SUBCELLULAR DOMAINS

Typical epithelial cells are surrounded by a plasma membrane that is divided into discrete domains, the

apical surface bearing channels, carriers, and other membrane proteins distinct from those along the basolateral surface. The respective domains are separated by the junctional complex, including a girdle of tight junctions situated along the lateral portion of the cell close to the luminal surface, which serves as a barrier to movement of proteins and lipids within the plane of the membrane from one domain to the other (Alberts et al., 1989).

Not only are the membrane proteins different in these domains, but so also are the subapical cytoskeletal structures, condensed into a "terminal web" apposed to the apical membrane. There may be other distinctive morphological specializations peculiar to the apex as well, including microvilli and cilia, and these too are associated with cytoplasmic cytoskeletal elements.

Traffic from the Golgi apparatus within the cell is also polarized with respect to the apical and basal membrane domains, some vesicles moving only to the apex and others only to the basolateral region. Individual vesicles are addressed specifically to one or the other domain; that is, the traffic is not stochastic but depends, rather, on specific targeting signals (Rodriguez-Boulan and Powell, 1992).

Although not as simple as that seen in typical epithelial cells, regional differentiation also occurs in myelin-forming cells. The pattern here is less straightforward. In a mature myelin-forming cell whose sheath is "unrolled," there is no simple apex and base and no symmetric band-like junctional complex formed with neighboring glial cells. Indeed, individual myelin-forming cells are not interconnected by any kind of cell-cell junction. Rather, myelin-forming cells form conspicuous tight junctions and, to some extent, adhesive junctions between different portions of the *same* cell, and they also form asymmetric band-like junctions with the underlying axon (Livingston et al., 1973; Dermietzel, 1974a; Schnapp and Mugnaini, 1975).

This axoglial junctional region appears as a narrow strip, the "lateral belt," ~0.1 to 0.2 μm wide, along both lateral edges of the glial cell (Figures 41-1 to 41-9). The junction clearly divides the axolemma into domains (see below), but it does not divide the myelin-forming cell itself into domains, since these junctions occur only at the extreme edges of the sheath. The tight junctions on either side of the axoglial junctional strip (Figures 41-1, 41-7, and 41-8) may isolate the strip from the remaining glial membrane, however.

Subdivision of a cell into domains can be accomplished by other mechanisms as well and does not necessarily require tight junctions, as has been shown in neurons (Rodriguez-Boulan and Powell,

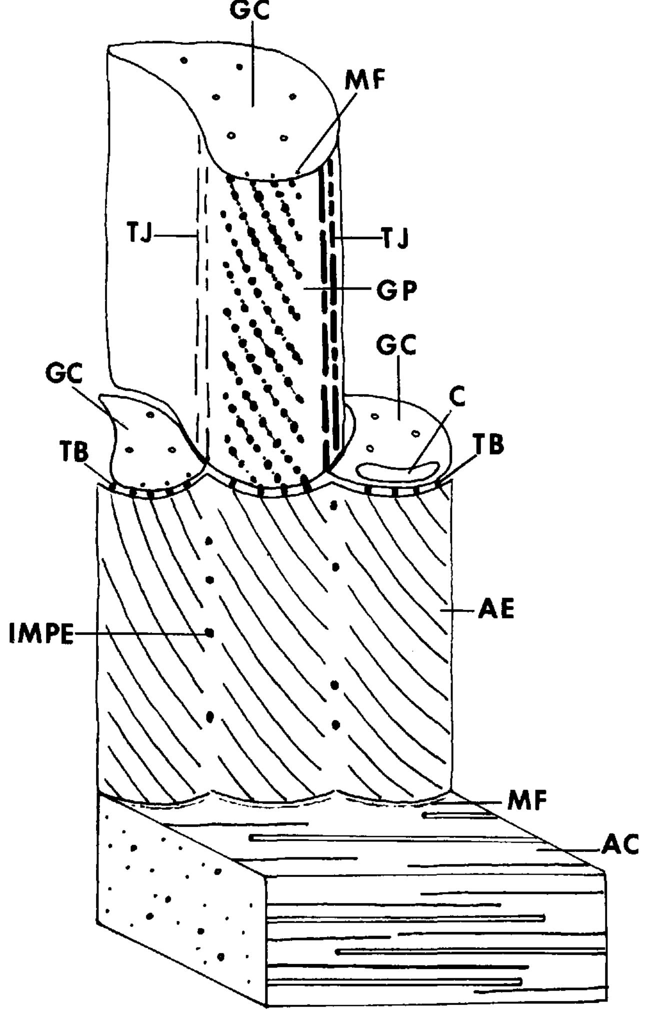

FIG. 41-1. Diagram of paranodal axoglial junction. The axonal axis is horizontal, with the nodal region toward the right (*not shown*) and the internode toward the left. Three "terminal loops" of glial cytoplasm (*GC*), containing circumferentially oriented microtubules, are shown. The P fracture face of the membrane of the middle loop displays diagonal rows of intramembranous particles between tight junctions (*TJ*). The intercellular junctional cleft of 2 to 4 nm is occupied by periodic ridges, the "transverse bands" (*TB*), which impress a diagonal pattern on the E fracture face of the axolemma (*AE*). Node-like intramembranous particles (*IMPE*) occupy rows in the axolemmal E fracture face between the indented strips of junctional membrane. The axonal cytoplasm (*AC*) contains axially oriented microtubules and neurofilaments as well as microfilaments (*MF*) immediately adjacent to the junctional membrane. The cytoplasm in two of the glial loops contains orthogonally oriented cytoskeletal filaments, seen as dots (cf. Figure 41-6) immediately adjacent to the junctional membrane (*MF*). The loop nearest the node contains a membranous cisterna (*C*) apposed to the junctional membrane.

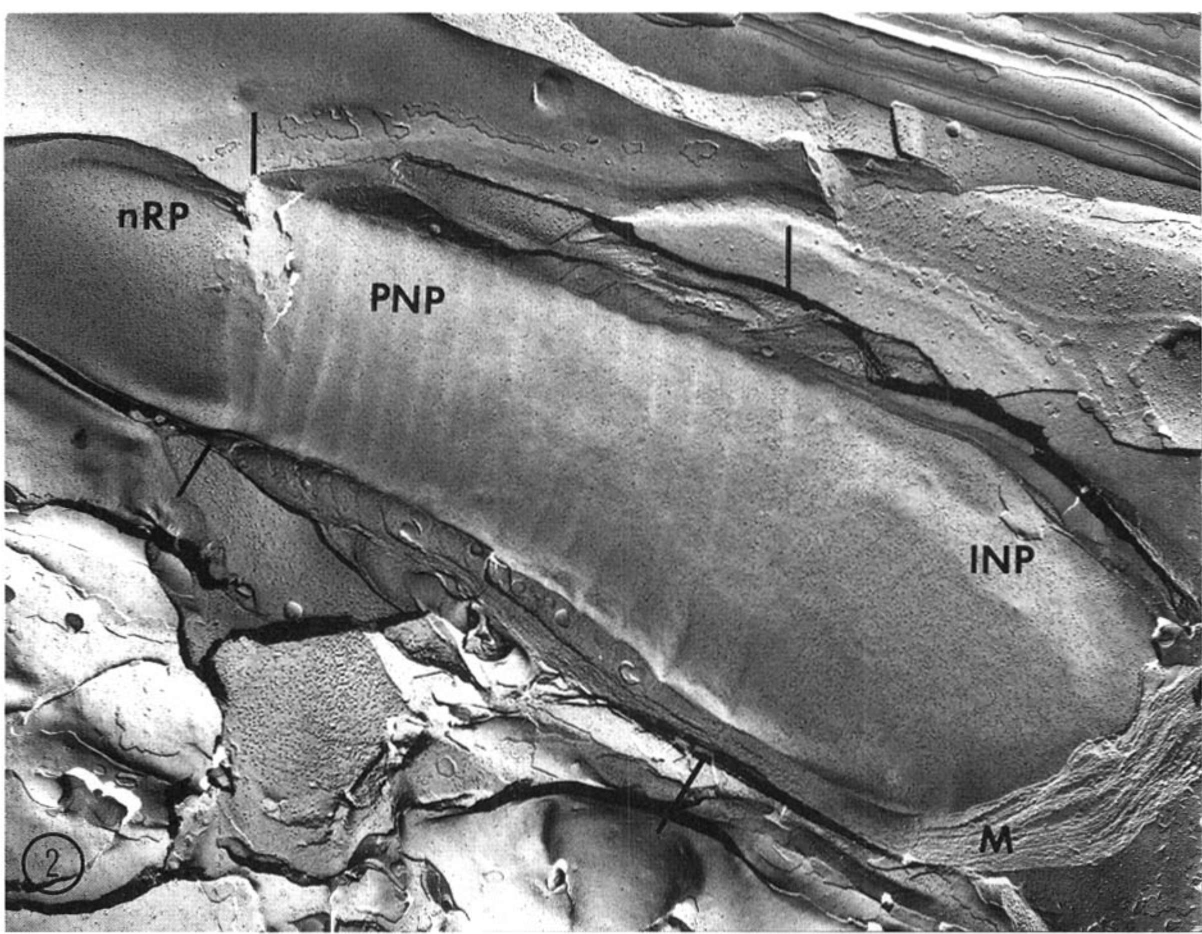

FIG. 41-2. Freeze-fracture replica of axon showing the inner leaflet (P fracture face) of the axolemma. The nodal membrane (*nRP*) is comparable to the internodal membrane (*INP*) in this fracture face. The paranodal region (*NP*) is marked by 17 strip-like indentations imposed by the apposed "terminal loops" of myelin lamellae. The loops themselves have been cross-fractured and are visible above and below the axolemma (*between bars*). *M*, compact myelin.

1992). In the case of myelin-forming Schwann cells, the outermost portion of the cell is enveloped by a basal lamina, which bridges across the node of Ranvier to the myelin segments on either side (Figure 41-8). Although the presence of a basal lamina would seem to identify the outermost layer of the Schwann cell as the base, the perinodal region of the cell gives rise to microvillous projections comparable to those normally arising from the apical domain in epithelial cells (Figures 41-8 and 41-9).

The innermost Schwann cell lamella and the "outer" perinuclear portions of the cell are separated by the great expanse of the intervening compact myelin. The enormous distance between the respective regions would seem to isolate them from each other at least partially. However, cytoplasmic "rivers," or channels, traverse the compact myelin and interconnect the innermost and outermost layers of the sheath (Gould, 1990). The most conspicuous, and consistent, of these transverse interconnections is represented by the *terminal loops,* which constitute the lateral margins of the sheath spiraling around the axon in the paranodal regions (Figures 41-1, 41-4, 41-6, 41-7, and 41-8). Other such channels, corresponding to *Schmidt-Lanterman incisures,* follow a similar spiral path at irregular intervals within the internodal myelin sheath. These various pathways serve as a potential route for movement of cytoplasmic components, including organelles and even mRNA (Colman et al., 1982; Trapp et al., 1987).

Although these pathways are extensive in peripheral myelin formed by Schwann cells, they are much more restricted in oligodendrocyte myelin in the central nervous system. Cytoplasm-containing channels corresponding to Schmidt-Lanterman incisures, occur in large-caliber sheaths (Hildebrand et al., 1993), but not in small-caliber sheaths. In all sheaths, however, regardless of size, the paranodal loops form a cytoplasmic channel along the lateral edges of each

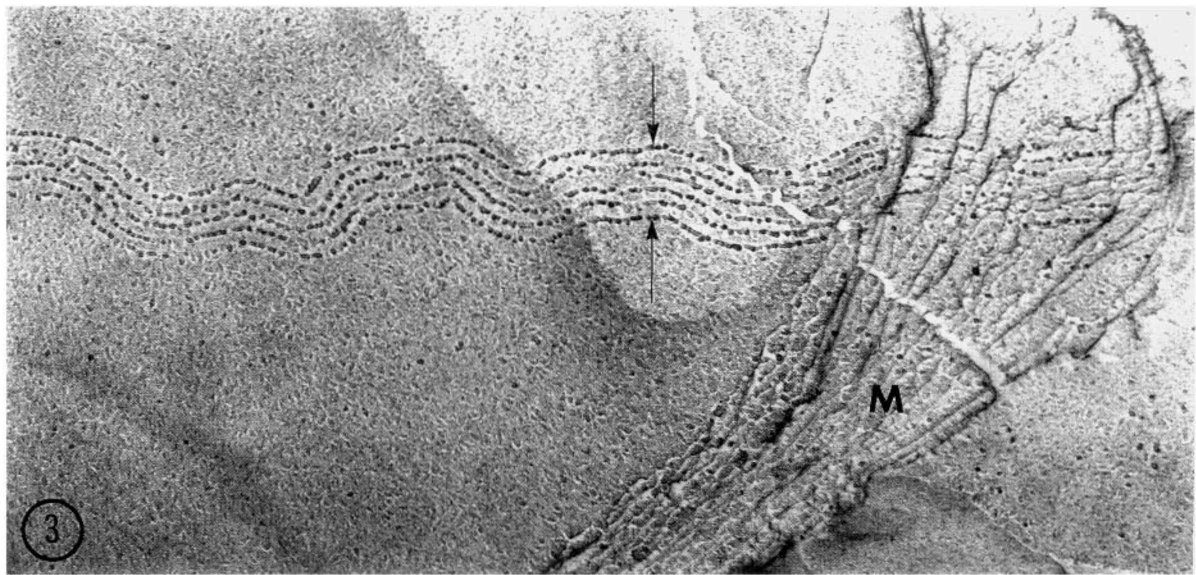

FIG. 41-3. The fracture face of a compact myelin lamella (left and center) contains five meandering "radial component" strands (*between arrows*). At right, the fracture steps through ~10 myelin lamellae, all of which display equivalent strands in register with those at left.

myelin segment continuous with the cytoplasm-containing innermost and outermost layers of the sheath. In some cases the outermost layer includes the oligodendrocyte cell body, and in other cases it is interconnected with the cell body by a narrow cytoplasmic process (Remahl and Hildebrand, 1990a; Peters et al., 1991).

Thus, even in the absence of zonular tight junction barriers, both peripheral and central myelin-forming cells can be roughly divided into the compact region of the myelin sheath, whose structure and biochemical composition are distinctive, and the cytoplasm-containing borders, which are continuous around the cell periphery, together with the transverse channels connected to them. Even within these regions, there appear to be subdomains. The lateral borders bear the paranodal axoglial junctional strips, flanked by tight junctions. The protein MAG (myelin-asso-

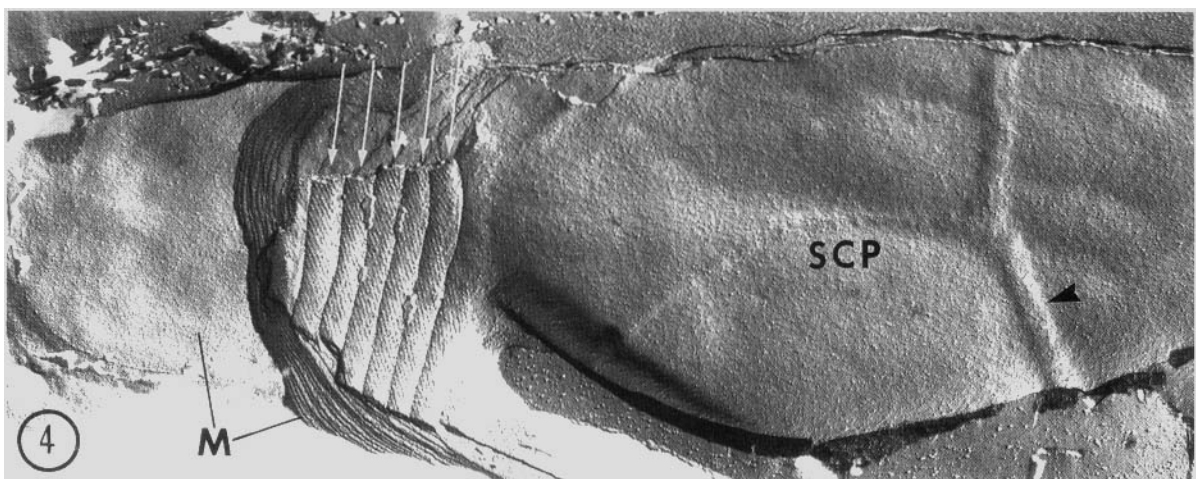

FIG. 41-4. Peripheral myelinated nerve fiber fractured to reveal the P-face of paranodal "terminal loops" (*arrows*) containing highly regular, diagonal rows of particles. The loop closest to the center of the figure is continuous with a myelin lamella (*SCP*), extending toward the right. Particles in the latter membrane are comparable to those in the loops, but are not organized into the same paracrystalline array. *Arrow* indicates the location of a cytoplasmic "channel" corresponding to a Schmidt-Lanterman incisure. Endoneurial connective tissue is visible at the upper left. *M*, additional myelin lamellae.

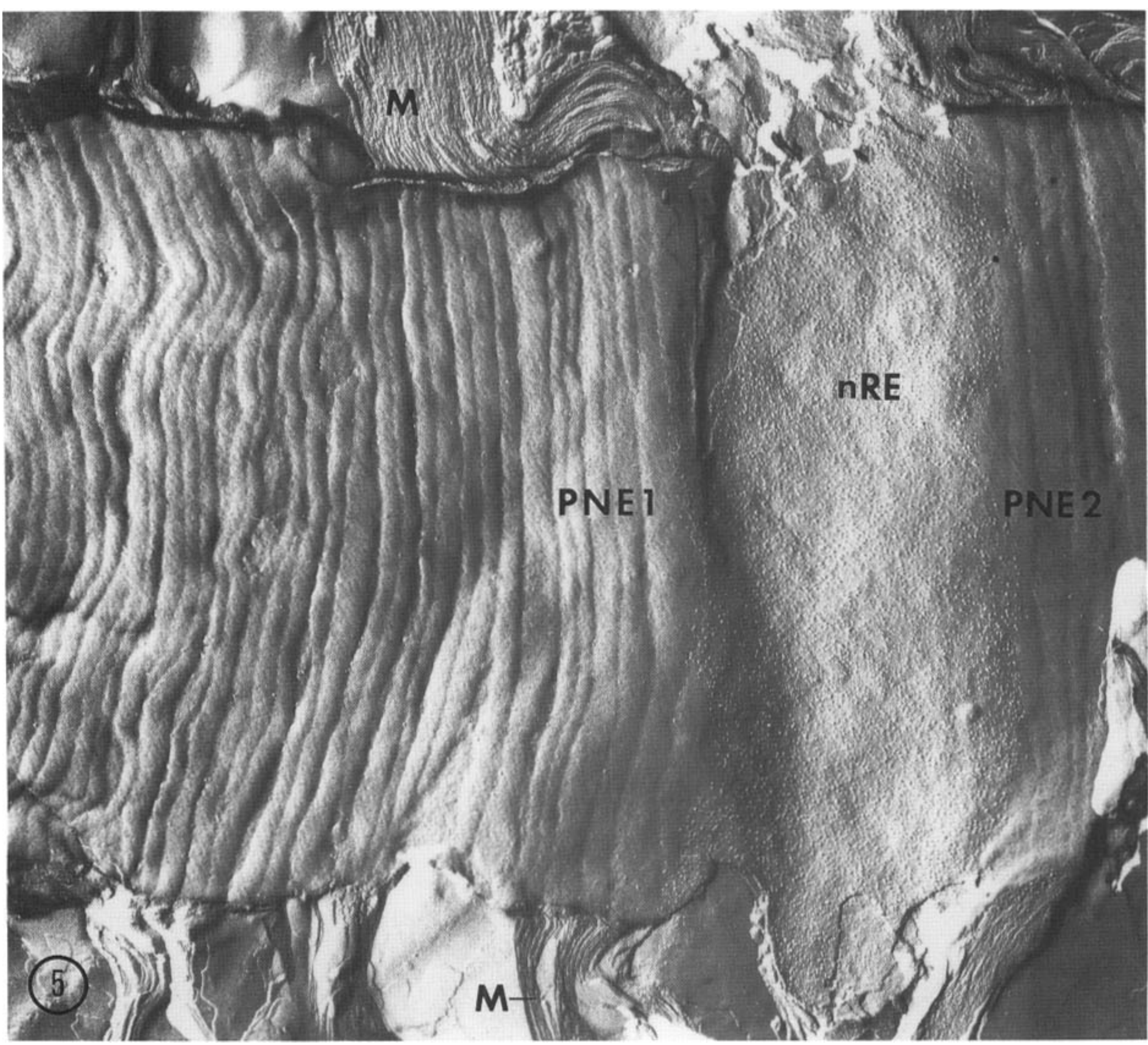

FIG. 41-5. Peripheral myelinated axon fractured to reveal the outer leaflet of the axolemma (E fracture face). The node of Ranvier (*nRE*) contains large numbers of membrane particles, in contrast to the internodal axolemma (*not shown*), which in this fracture face contains very few. The nodal particles are sharply demarcated by the paranodal regions on either side (*PNE1* and *PNE2*), which display multiple strip-like indentations bearing a diagonal pattern and few membrane particles, except within "grooves" (upper right). There are ~33 indentations in PNE1, representing junctions with terminal loops such as those shown in Figure 41-4. *M*, compact myelin.

ciated glycoprotein) appears primarily in association with the innermost border, corresponding to the first layer of the sheath, adjacent to the enclosed axon, plus, in Schwann cells, the Schmidt-Lanterman incisures and paranodal terminal loops (Trapp, 1990).

The extent to which membrane components are able to move from one domain to the next within the plane of the membrane is unknown. Nor is it clear how turnover of compact myelin components is accomplished. One can only speculate that membrane elements diffuse laterally within the plane of the membrane to the nearest cytoplasmic channel, where "packages" of new components transported from the perikaryon via cytoplasmic vesicles are available for replacement of old components, which are then transported back to the perikaryon. The possibility remains that the myelin sheath "grows" continuously from the outer edge, nearest the perikaryon, and is sloughed continuously from the innermost edge. This conflicts with the observation that different components of myelin turn over at different rates, however (Norton, 1981). Some components may also be able to move transversely across lamellae, and some may be synthesized by enzymes resident within compact myelin (Ledeen, 1984).

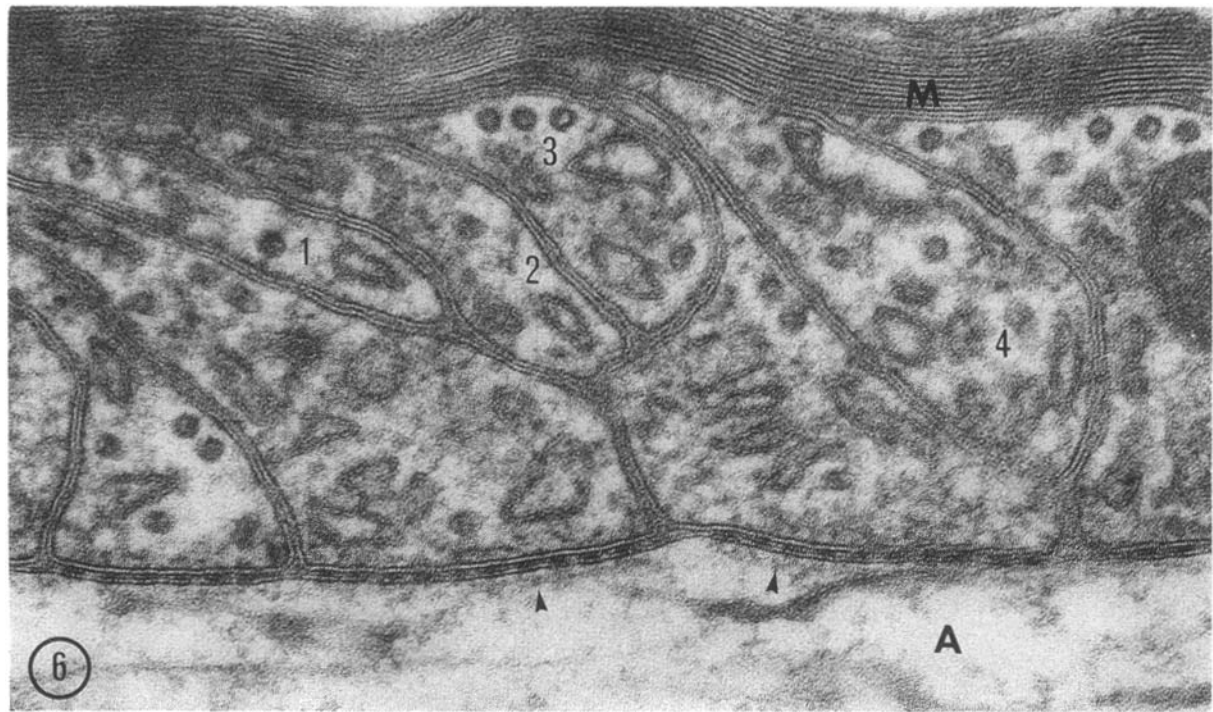

FIG. 41-6. Thin section of paranodal region. Compact myelin lamellae (*M*) split to form tear-drop-shaped "terminal loops" that extend toward the axon (*A*). Some of the loops form junctions with the axolemma, marked by periodic densities, the "transverse bands." In the axoplasm just beneath each junction, microfilaments (*arrowheads*) run parallel to the axolemma. Fine dots in the terminal loop cytoplasm just adjacent to the junctional membrane represent orthogonal microfilaments cut transversely. The terminal loops contain membranous cisternae and microtubules. Four of the loops (1 to 4) do not reach the axolemma and display no junctional specializations. Tight junctions are evident between terminal loop membranes.

GLIA-GLIA JUNCTIONS

Although in typical epithelial cells the subapical junctional complex between adjacent cells appears to constitute a dividing line between apical and basolateral domains, myelin-forming cells, as pointed out above, have no equivalent junction with their immediately adjacent fellows. At the extreme ends of each internode in the peripheral nervous system, adjacent Schwann cells abut each other, but they do so in a spray of microvillous processes that intermingle and approach each other closely, but do not, so far as is known, form junctions with each other (Figures 41-8 and 41-9). The Schwann cell-Schwann cell interaction at the node of Ranvier is thus distinct from the epithelial cell-epithelial cell junction at the junctional complex.

The latter junction, in addition to separating apical from basolateral domains within each cell, serves to isolate the extracellular fluid surrounding the apices of the cells from that surrounding their bases, which may be very different in composition. In the case of a myelinated axon, however, the fluid bathing the outside of the myelin sheath is directly continuous with that bathing the node of Ranvier. Only a basal lamina separates the two. Indeed, saltatory conduction requires a low-resistance pathway extending from the nodal axolemma to the extracellular space surrounding the internode. Any barrier to current flow here would undoubtedly disrupt saltatory conduction.

Within the central nervous system, myelin-forming oligodendrocytes do not form microvilli over the node of Ranvier. The equivalent structures in this case are either absent or are formed by astrocytes (Hildebrand, 1971), again with no high-resistance barrier between the axonal surface at the node and the remainder of the extracellular space.

In contrast to the lack of specialized junctions between neighboring myelin-forming cells, myelin lamellae within a single internode, derived from the same cell, form very conspicuous tight junctions between successive terminal loops in the paranodal regions (Figures 41-1 and 41-6). These junctions serve to isolate the minute paranodal periaxonal space from the narrow aqueous space between successive layers of the sheath. Similarly, the internal mesaxon, which constitutes a potential pathway interconnecting the internodal periaxonal space with the extracellular space within the sheath, is also

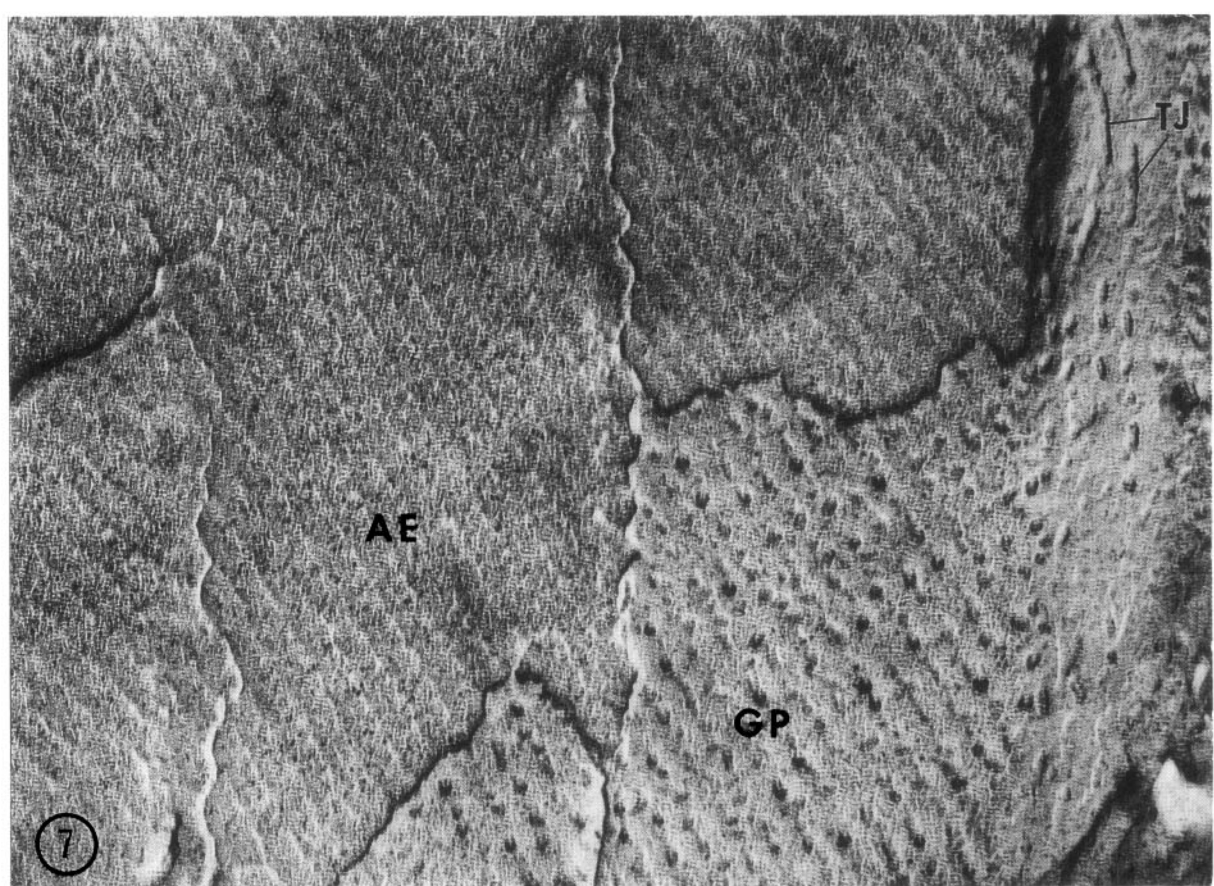

FIG. 41-7. Freeze-etched specimen showing the external leaflet of the paranodal axolemma (*AE*) dried down against "terminal loops," whose cytoplasmic leaflet (*GP*) displays paracrystalline rows of membrane particles having the same orientation and repeat period as the pattern in the axolemma. The junctional face of the terminal loop membrane is flanked by tight junction strands (*TJ*).

sealed closed by tight junctions (Figure 41-8), as is the external mesaxon formed between the outermost layer of the sheath and the one just internal to it (Tetzlaff, 1978; Dermietzel et al., 1980; Shinowara et al., 1980), thus apparently isolating the extracellular space within the sheath from that outside. The disposition of these tight junctions is such that the compact region of the myelin sheath is ringed by tight junctions, laterally, internally, and externally. Presumably, the seal is effective over the brief duration of an action potential, although it can be penetrated over longer time periods by cations or hypo-osmolar media (Ropte et al., 1990) and by antibodies (Raine et al., 1978).

To what extent the tight junctions extend further, into the compact region, is uncertain. Particle strands (Figure 41-3) associated with the *radial component* (Kosaras and Kirschner, 1990) extend across multiple myelin lamellae, primarily in the quadrant that includes the inner and outer mesaxons (Peters et al., 1991). It is not known whether these have the same significance as tight junctions in restricting movement through the extracellular compartment within the myelin sheath, either circumferentially or longitudinally. Nor is it clear whether the radial component strands serve to divide the compact membrane domain by restricting movement of membrane components within the plane of the myelin membrane.

Adhesive junctions are also seen sometimes between successive terminal loops, that is, between different portions of the same cell membrane, but not between different myelin-forming cells at adjacent internodes. Typically, the adhesive junctions are found adjacent to the tight junctions on the side away from the axolemma in a position corresponding to that of the girdle-like adherent junctions at epithelial junctional complexes. Presumably, they serve to anchor the respective terminal loops together mechanically and to prevent slippage between successive layers of the sheath in the paranodal region. In the unrolled state, these adherent junctions

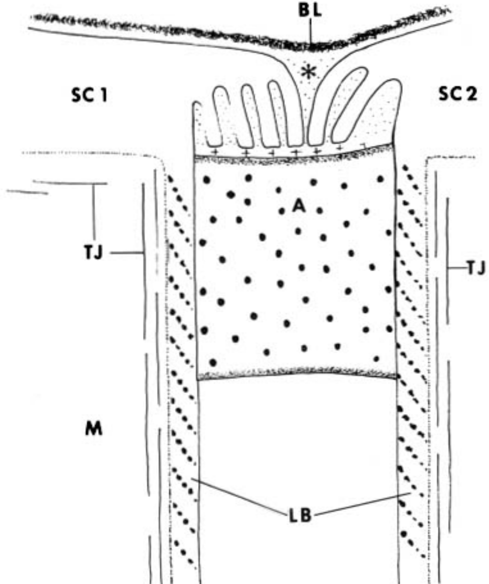

FIG. 41-8. Diagram of nodal junction between "unwrapped" Schwann cells (*SC1* and *SC2*) and an axon (*A*) displaying a typical "undercoating" of the nodal axolemma. A basal lamina (*BL*) bridges across the nodal gap, which contains a proteoglycan "gap substance" and many slender processes arising from the adjacent Schwann cells. The processes stabilize at a constant separation of ~20 to 25 nm from the axolemma; this space contains both transverse and parallel junctional filamentous structures. Adjacent to the node, the unwrapped lateral belt of each Schwann cell is marked by diagonal particle arrays, which form the paranodal axoglial junction (*not shown;* see Figure 41-1). Tight junctions (*TJ*) are present between the compact myelin (*M*) and lateral belts and between the Schwann cell cytoplasm and outermost layer of compact myelin.

would appear intermittently in the membrane of the terminal loops adjacent to the tight junction strands.

A recent confocal microscopy study of peripheral nerve fibers has demonstrated E-cadherin associated with the plaque-like adhesive junctions, aligned in register between successive paranodal loops of myelin, as well as with the internal and external mesaxons and Schmidt-Lanterman incisures. This protein colocalizes with β-catenin and F-actin, suggesting that the complexes thus formed may serve not only to maintain the integrity of the cytoplasmic channels within the myelin sheath and the organized structure of the sheath, but also to transmit signals across myelin lamellae (Fannon et al., 1995).

Although oligodendrocyte processes of the same cell are known to form tight junctions with each other, tight junctions between astrocytes and oligodendrocytes have not been reported (Mugnaini, 1986). Rather, gap junctions form between the respective cells. These are macular structures that do not encircle membrane domains or isolate extracellular spaces. If the perinodal astrocyte is indeed involved in the regulation of extracellular ion composition, as has been proposed, coupling to oligodendrocytes by way of gap junctions might serve to redistribute ions intracellularly within oligodendrocytes as well as astrocytes. Mechanical adhesion might also be postulated, that is, a mechanism for immobilizing and retaining astrocytes at the perinodal locus. In addition, the passage of small molecules involved in signaling, for example, cAMP, could couple the respective cells into a *metabolic syncytium* and serve to coordinate their behavior (Ransom and Kettenmann, 1990; Chapter 19, this volume).

PARANODAL AXOGLIAL JUNCTIONS

More striking than the gap and tight junctions formed by myelin-forming cells are the "close" axoglial junctions, which are formed by the lateral edges of the sheath as it winds around the axon (Livingston et al., 1973; Dermietzel, 1974a; Schnapp and Mugnaini, 1975). The winding pattern is distinctive. In the case of a roll of paper, all the layers are coextensive, and only the innermost one is in contact with the core. In the case of the myelin sheath, however, successive layers are slightly shorter, moving from the outside of the sheath inward, and, as a result, the edge of *each* turn of the sheath is in contact with the axolemma (Figures 41-4 and 41-12). Hence the total area of contact between the sheath and the axon is enormous, the amount increasing with the number of turns and the diameter of the axon.

Since the width of each junctional strip is ~0.1 to 0.2 μm, 20 adjacent strips at a single central nervous system paranode would extend up to 4 μm in the axial direction (Figure 41-12). For an axon 2 μm in diameter, the total surface area of one such paranodal junction would approach ~24 μm^2. Since there are two such regions for each myelin segment, the total area of axoglial junction in this segment would be almost 50 μm^2. In the case of peripheral nervous system fibers (Figure 41-5), the number of turns of the myelin sheath can be much greater, and the diameter of the axon in the paranodal region may be greater as well, resulting in an increase in junctional area, although in the case of very thick sheaths, not all terminal loops reach the axon (Figure 41-6). Nevertheless, the paranodal axoglial junction is surely the largest intercellular junction known and presumably subserves one or more important functions.

This junction is not only extraordinary in size, but also unique in form, with no clear counterpart in other tissues. Its distinctive characteristics include the following:

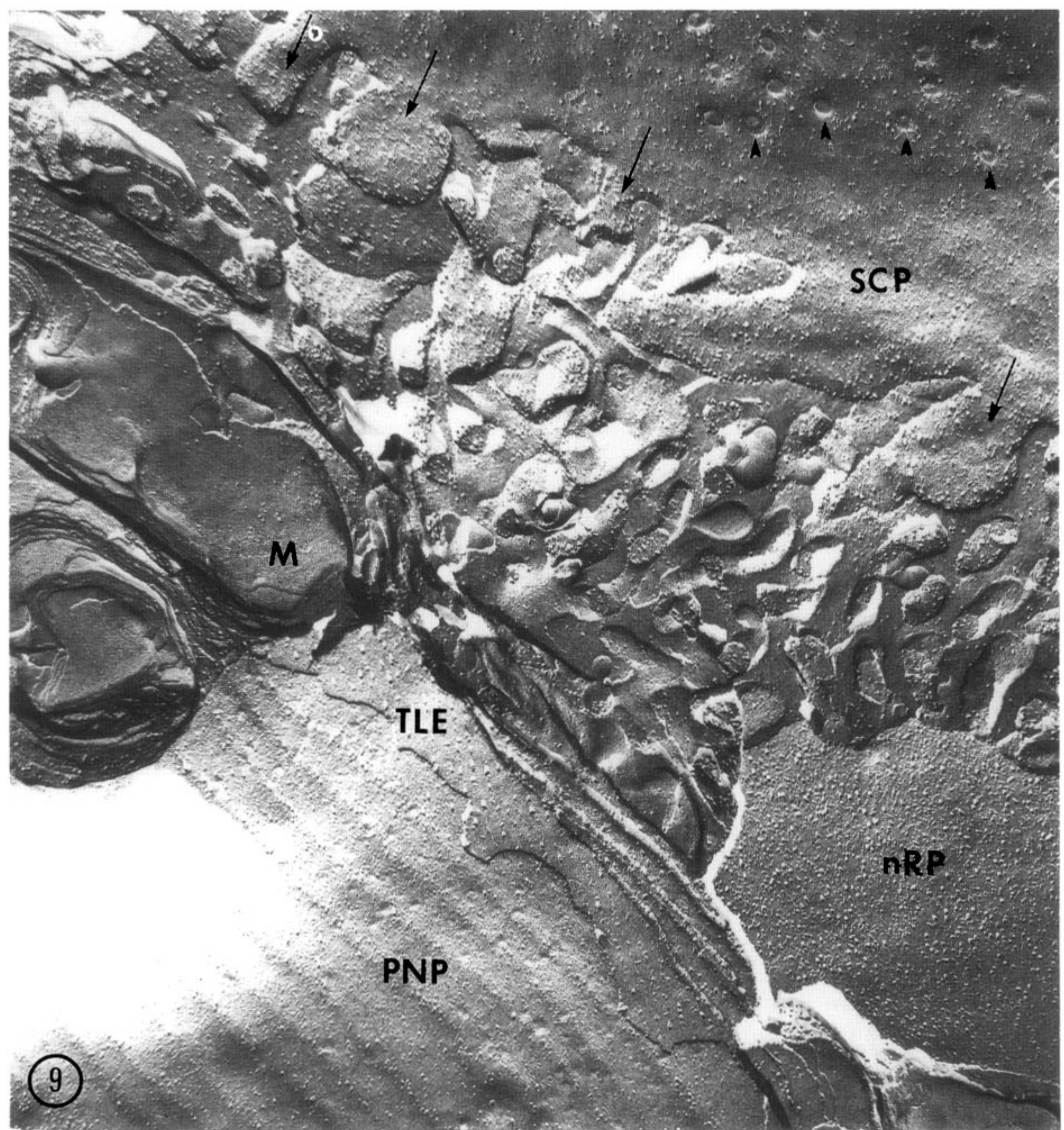

FIG. 41-9. Freeze-fracture replica of peripheral node of Ranvier. The P fracture face of the paranodal axolemma (*PNP*) containing strip-like junctional indentations is shown at the lower left. It is partly covered by terminal loop membrane (*TLE*), which in the E fracture face displays few particles, but has a faint diagonal pattern. The P face of the node (*nRP*) is covered by a profusion of microvilli within the perinodal space in the center of the figure. Some of these can be seen to arise from the adjacent Schwann cell (*SCP*) at *arrows*. *Arrowheads*, pinocytotic pits. *M*, myelin.

1. Exceptionally close apposition (2 to 4 nm separation) between the axolemma and the apposed terminal loop membranes (Figure 41-6), comparable to the membrane spacing at gap junctions (Livingston et al., 1973)
2. Indentation of the axolemma (Figures 41-2, 41-5, 41-6, and 41-9) by the terminal loops (Robertson, 1959)
3. Ordered, oblique arrays (Figures 41-1, 41-4, and 41-7) of membrane particles within the junctional membrane of the terminal loop (Livingston et al., 1973; Rosenbluth, 1984)
4. Oblique intercellular ridges (Figures 41-1 and 41-6), the *transverse bands* (Hirano and Dembitzer, 1982), corresponding in orientation and period to the terminal loop particle arrays

5. Filamentous cytoskeletal elements (Figures 41-1 and 41-6) in both axon and terminal loop cytoplasm (Rosenbluth, 1984, 1990; Ichimura and Ellisman, 1991). In addition, membranous cisternae (Figure 41-1) occur commonly in the terminal loops in close apposition to the junctional membrane (Rosenbluth, 1984), but these are not consistent, and in the central nervous system they are uncommon.

The axoglial junction is exclusively heterophilic; that is, Schwann and glial cells never form such junctions between their own processes or with adjacent cells of like kind. "Floating" terminal loops that do not reach the axon show no sign of junctional or hemijunctional structure (Figure 41-6). Nor do axons form such junctions with other neuronal processes. Presumably, neither myelin-forming cells nor neurons are capable of producing all the components necessary for assembly of this junction. Indeed, only the axonal portion of the neuron appears capable of forming this junction.

Although the prodigious size of this junction suggests that it subserves one or more important functional roles, what those roles are is still uncertain. Several possibilities seem likely.

Anchorage of the Myelin Sheath. The junction may serve to anchor the myelin sheath to the axon; that is, transmembrane components may attach the cortical cytoskeleton of the axon to that of the glial cell in the paranodal region, thus maintaining the nodal gap at a fixed length regardless of extraneous mechanical stresses. This would be of most importance in the peripheral nervous system, in view of the considerable lengthening and shortening of nerves associated with skeletal movements. Clearly, changes in nodal area would have serious effects on nodal capacitance and safety factor and could cause conduction failure.

Electrical Isolation. The junction may serve to isolate the perinodal space between myelin segments from the periaxonal space underneath the myelin sheaths electrically (Rosenbluth, 1976, 1983). The action currents generated at the node flow axially within the axon and longitudinally outside the myelin sheath, forming circuits completed at adjacent nodes of Ranvier. If a substantial part of that current were to be short-circuited underneath the myelin sheath, the effectiveness of saltatory conduction would be compromised. Thus, it is essential that the paranodal myelin sheath be sealed to the axon in such a way as to impose a high resistance to current flowing longitudinally along the external surface of the axon.

The simplest biological device to meet this need would be a tight junction between the glial terminal loops and the axolemma. In fact, this does not occur. Tracer molecules are able to follow this pathway (Feder et al., 1969; Hirano and Dembitzer, 1969), and, in addition, some current flow has been reported through this pathway after completion of action potentials, apparently representing the discharge of internodal capacitance (Barrett and Barrett, 1982). Thus, the characteristics of this junction appear to be such that the high-frequency current flows that accompany the action potential are not significantly short-circuited, while at the same time, slower time-course currents associated with afterpotentials are able to flow.

Perhaps more important, there may be sufficient electrical continuity through the junction so that internodal potassium channels are able to establish the resting potential for both the nodal and internodal axolemma (Chiu and Ritchie, 1982), since there may be relatively few potassium channels in the nodal membrane itself.

Differentiation of the Axolemma. Freeze-fracture studies of unmyelinated axons show the axolemma to be a relatively uniform structure containing randomly scattered particles without obvious regional differentiation (Figure 41-12 bottom). Myelinated fibers, in contrast (Figures 41-5 and 41-12 top) are differentiated in a highly distinctive manner with respect to the distribution of membrane particles in the external leaflet of the axolemma (Rosenbluth, 1976). Typically, the nodal axolemma, which has the form of a cylinder or barrel, depending on its curvature, contains large numbers of large particles, sharply demarcated by the collar of paranodal junctions on either side of the node. The paranodal axolemma is serially indented by the helically wound lateral edge of the myelin sheath, successive turns of which are closely apposed to each other (Figures 41-4 and 41-12 top).

In contrast to the nodal axolemma, the paranodal axolemma contains only small numbers of node-like particles, and those present occur primarily within the narrow "grooves" between junctional strips. Occasionally, especially in that portion of the paranode furthest from the node, the strips are less tightly packed, and the grooves between them widen into "lakes," which contain larger numbers of node-like particles. Such particles occur as well in the juxtaparanodal portion of the internodal axolemma (JPI) immediately adjacent to the paranode. The particles here are sharply demarcated by the last junctional strip of the paranode, their concentration declining gradually with distance in the direction of the internode to a baseline level of ~$100/\mu m^2$ (Tao-Cheng and Rosenbluth, 1984).

The E-face of the paranodal junctional axolemma often displays a paracrystalline pattern (Figures 41-5 and 41-7), especially in specimens that have been etched, that is, those in which the water between the axolemma and apposed glial membrane has sublimed, resulting in collapse of the outer leaflet of the axolemma against the intercellular, ridgelike transverse bands (Rosenbluth, 1984). The result is that the image of the transverse bands becomes embossed on the junctional axolemma, resulting in the appearance of a diagonal pattern within the strips. In some preparations, the inner leaflet of the paranodal axolemma displays a complementary pattern, which could also have been embossed by the transverse bands prior to fracture. This pattern has been ascribed, alternatively, to arrays of particles intrinsic to the paranodal axolemma (Wiley and Ellisman, 1980). However, the absence of such a pattern in acutely demyelinated axons does not support that interpretation.

It is clear that the angle formed by the paranodal transverse bands in mammalian nerves approaches 90 degrees with respect to the axonal axis, but in amphibian (Rosenbluth, 1976; Tao-Cheng and Rosenbluth, 1980) and reptile nerves (Schnapp and Mugnaini, 1975) the angle is obviously smaller. Presumably, the orientation of the particles in the junctional glial membrane determines this angle.

Intercellular Transfer. The great length of the axon has suggested that this appendage, particularly where it is most remote from the parent neuronal perikaryon, may depend on the investing glia for some sort of "nutrition" or "support." Most recently, this hypothesis has taken the form of suggestions that voltage-gated sodium channels may be generated in glial or Schwann cells and subsequently transferred to axons by way of the nodal microvilli (Shrager et al., 1985). The paranodal junction would be another possible site for transfer in view of its size, the much closer proximity of the axon and glial membranes to each other here, and the strategic location of the junction with respect to the node of Ranvier.

Although provocative, the hypothesis that glial channels are transferred to axons has not yet received direct support, however. Indeed, recent data (see *Pathology* below) suggest that axolemmal sodium channels in the central nervous system of the myelin-deficient rat, which is virtually devoid of myelin, as well as the *shiverer* mouse, are not reduced. On the contrary, evidence from both freeze-fracture studies and saxitoxin-labeling experiments suggests that myelin deficiency results in an overall *increase* in axonal sodium channels, with higher-than-normal concentrations in the axolemma.

The converse possibility, that the paranodal junction is a site of signaling from the axon to the myelin-forming cell, remains open, although it must be pointed out that the junction has not yet formed during the early stages of myelination, but appears only later (Tao-Cheng and Rosenbluth, 1983). Nevertheless, the junction could be the site at which the mature myelin-forming cell "reads" the state of the enclosed axon. The junction could thus underlie phenomena such as Wallerian degeneration, in which myelin segments far from the site of an axonal injury begin to degenerate, even though they have not been directly damaged by the trauma (Ishise and Rosenbluth, 1986).

NODAL AXOGLIAL JUNCTIONS

The nodal axolemma also bears a characteristic relation to the surrounding Schwann or glial cell processes, which is distinctly different from that in the paranodal region and is sufficiently specialized in structure to justify calling it a junction. The essential character of this junction can be seen not only at normal nodes of Ranvier in both the peripheral nervous system (Figure 41-8) and central nervous system (Figure 41-12), but also, and in some cases to better advantage, in abnormal fibers (see *Pathology* below).

The essential features of the nodal junction include, on the axonal side, a distinct "undercoating" (Peters et al., 1991) of the plasma membrane by cytoskeletal elements (Figures 41-1, 41-8, 41-10, and 41-11). An isoform of ankyrin has been localized to this site (Kordeli et al., 1990). The external surface of the nodal axolemma is separated from the surrounding glial processes by a space having a consistent width of ~20 to 25 nm (Figures 41-10 and 41-11), that is, ~5 to 10 times the width of the paranodal gap. This space is reminiscent of that at desmosomes or adhesive junctions between epithelial cells not only in size but also with respect to the presence of a dense lamina within the cleft and irregular interconnections between the axonal and glial membranes (Figures 41-8, 41-10, and 41-11). These fine filamentous interconnections are visible both in thin sections (Rosenbluth, 1983) and in etched preparations (Ichimura and Ellisman, 1991).

In those cases where the external glial component of the junction is missing, the axonal components are nevertheless present, and the complex resembles the *hemidesmosomes* that link intracellular cytoskeletal elements to extracellular fibrous components, as

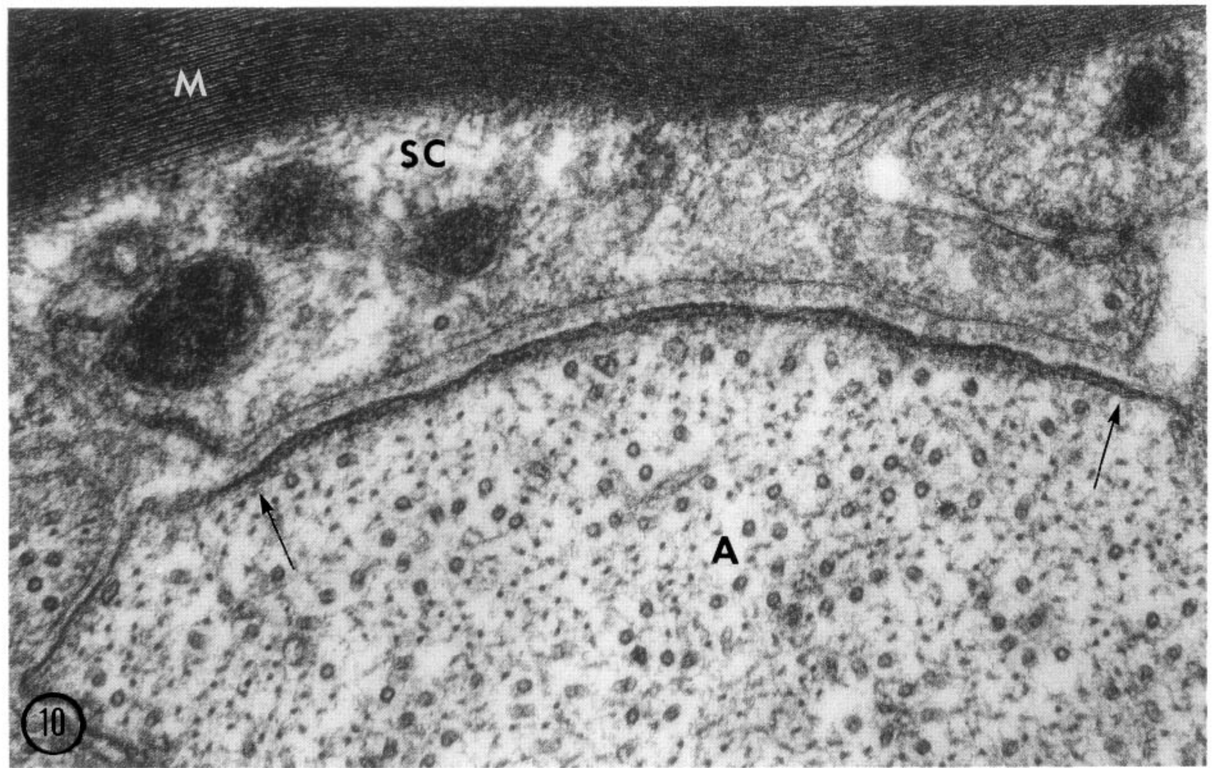

FIG. 41-10. Dystrophic mouse spinal root axon (*A*) forming an aberrant node-like junction (*between arrows*) with a Schwann cell (*SC*), which has formed a compact myelin sheath (*M*) around another axon (*not shown*). The junction is marked by an axoplasmic undercoating and cross-shaped filaments in the intercellular junctional space. The respective membranes follow each other closely at a separation of ~20 to 25 nm.

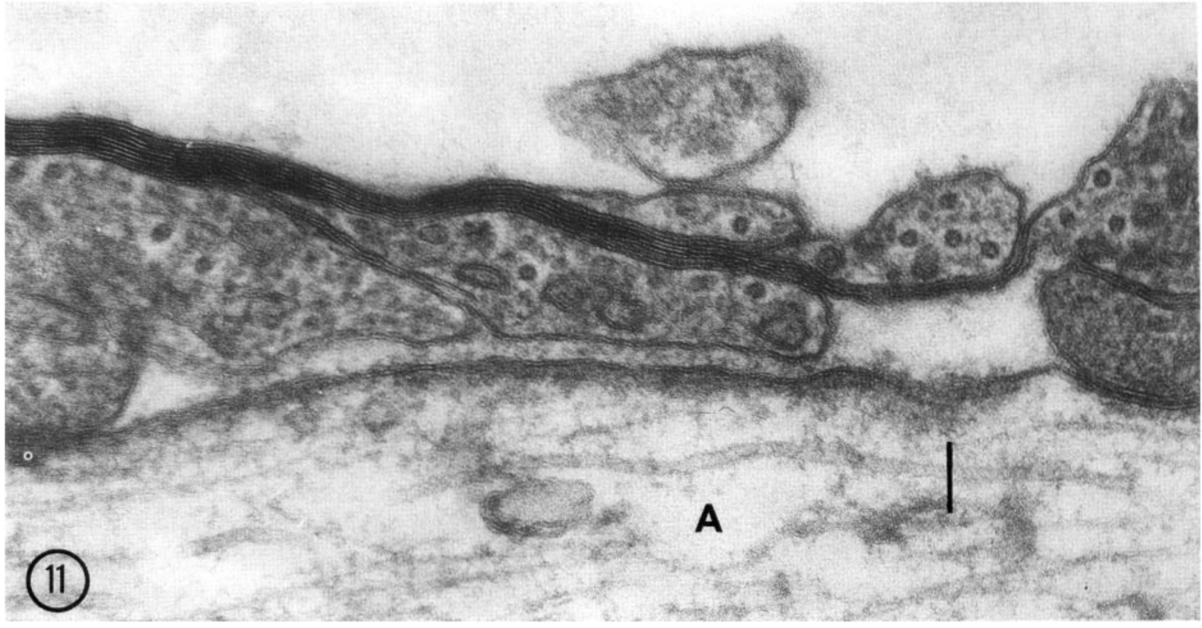

FIG. 41-11. Axoglial junctions formed between transplanted oligodendrocytes and spinal cord axon (*A*). To the right of the bar, and at the extreme left of the figure, the junctions are of the close paranodal type. To the left of the bar, the junctions are node-like, with an axolemmal undercoating and a wide gap containing intercellular filamentous material.

in the case of epithelial cells facing a basal lamina adjacent to a connective tissue matrix, or muscle cells at a myotendinous junction.

In the peripheral nervous system the nodal junction is formed by the same Schwann cells that form the adjacent myelin segments, but nodal junctions in the central nervous system are typically formed by astrocyte processes, not oligodendrocytes (Hildebrand, 1971). Occasional oligodendrocyte-axon junctions may nevertheless be seen at central nervous system nodes of Ranvier. These too show the typical wide but uniform separation of membranes, intercellular density, and axolemmal undercoating (Figure 41-11).

The functions of the nodal junction are uncertain. Adhesion is obviously one, especially in view of the localization of adhesion molecules to that site (Waxman, 1986; Salzer, 1995). In addition, a role for the perinodal glial processes in nodal differentiation has been postulated, although it is not difficult to find central nervous system nodes with little or no astrocytic investment, both during myelinogenesis and in adults. In the latter case, the undercoated nodal membrane faces a widened extracellular space containing just the extracellular proteoglycan matrix.

Observations made on glial cell-deficient spinal cord, in which both oligodendrocytes and astrocytes are sparse, suggest that the nodal axolemma (or elements in its vicinity) *induces* astrocytes to extend processes toward the perinodal region, but leave open the converse possibility, that is, that astrocyte processes may be involved in *determining* nodal sites (Sims et al., 1985). Even if the nodal axoglial junction proves not to be involved in the initial differentiation of the nodal axolemma, it may nevertheless play a significant role in the *subsequent* stabilization and maintenance of the differentiated nodal axolemmal structure (see below).

Node-like junctions also form in the nonmyelinated portion of the retina between retinal ganglion cell axons and Müller cell processes. Here, partial node-like specializations, including an axolemmal undercoating that extends part way around the axonal circumference (Hildebrand and Waxman, 1983), and node-like membrane particle patches of aberrant shape (Black et al., 1984) develop in the axons in the absence of myelin or myelin-forming cells. Similar observations have also been made in fibers lacking myelin as a result of pathological processes (see *Pathology* below). In all these cases, it is clearly impossible to invoke the paranodal junction or myelin-forming cell as an essential factor in nodal differentiation, since the perinodal glial component of the nodal junctional complex alone is associated with the differentiated state of the membrane. In nerve fibers lacking glia entirely, however—for example, at the center of an ethidium bromide lesion (see below)—there is no convincing evidence that any new node-like specializations form or that those previously formed are permanently maintained (Rosenbluth et al., 1985).

POSSIBLE MECHANISMS OF GLIAL INFLUENCE ON AXOLEMMAL STRUCTURE

The paranodal axoglial junction divides the axolemma into discrete nodal and internodal domains, which differ markedly in structure and ion channel distribution. Figure 41-12 illustrates the characteristic distribution of E-face particles in the axolemma of a myelinated fiber at and around the node of Ranvier.

Several means can be proposed by which glial association could bring about nodal specialization. The structure of some integral membrane protein molecules, including the sodium channel, is such that they project substantially beyond the external surface of the axolemmal bilayer (Barchi et al., 1981). At close axoglial junctions of the paranodal type, apposition of glial processes to only 2 nm from the axolemma may obstruct insertion of such molecules into that region of the axolemma. In addition, close apposition of glial membranes to the external surface of the axolemma may sterically prevent membrane proteins of this kind in adjacent, *non*-junctional regions from moving laterally within the plane of the membrane through the junctional region. Thus, sodium channels could become "trapped" at a presumptive node flanked by close axoglial junctions. Ionic currents generated by small clusters of such trapped channels could in turn lead to the recruitment of new channels to the same site (Rosenbluth, 1984). The original cluster may thereby "nucleate" the development of a full-blown node.

Nodal integral membrane protein molecules could also become "trapped" through linkage to perinodal glial processes externally, although it is not clear that perinodal glial processes are consistently present during the earliest stages of nodal development. In addition, they could form clusters by becoming bound to each other and thus immobilized. However, freeze-fracture studies show nodal particles to be randomly distributed with no evidence of close packing or crystalline arrays. Finally, nodal particles could become bound to preexisting axonal cytoskeletal elements. However, particle clusters would then be expected even in the absence of interaction with glial cells, and, as discussed above, there is no con-

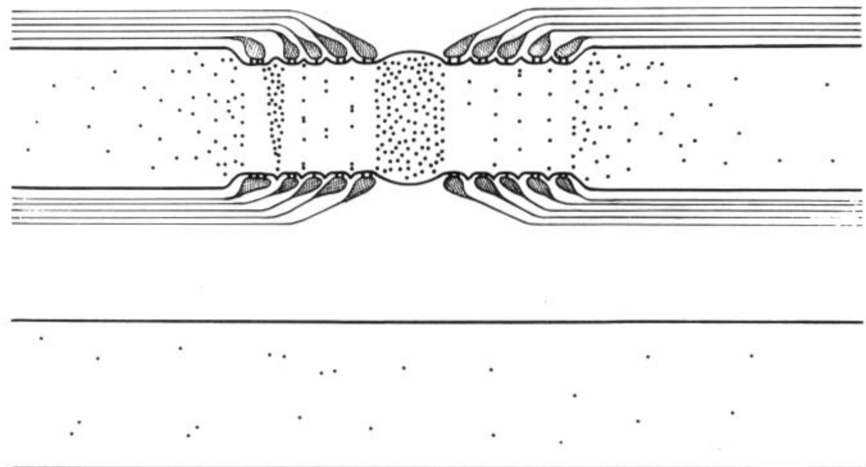

FIG. 41-12. **Top:** Diagram of nodal and paranodal regions of a myelinated axon in the central nervous system. The nodal particles characteristic of the axolemma, which are thought to represent sodium channels, are indicated by *black dots*. These are highly concentrated and randomly distributed at the node, in the center. They are also present in linear strings between junctional indentations in the paranodal regions, on either side of the node, and, in larger numbers, in an occasional "lake" (*left*) where the junctional indentations separate. Particle concentration in the juxtaparanodal region of the internode (*JPI*) is highest adjacent to the paranode and diminishes gradually in the lateral direction. In three dimensions, the paranodal particle "strings" are part of a continuous helical strip interconnecting the node with the JPI and represent a potential route for addition or removal of nodal sodium channels (see Rosenbluth, 1976). **Bottom:** Sparse, randomly scattered membrane particles in an unmyelinated axon. [From Rosenbluth (1989), with permission.]

vincing evidence that completely naked axons display node-like axolemmal specializations.

The essential role of glia in nodal differentiation has been confirmed in recent studies of cultured neurons showing a random distribution of axolemmal sodium channels, in the absence of Schwann cells, and clustering of sodium channels after addition of Schwann cells to the culture. This effect was independent of the distribution of the axonal cytoskeletal elements ankyrin and spectrin (Joe and Angelides, 1992).

In remyelination as well, sodium channel aggregates form initially at the edges of redeveloping myelin sheaths, then shift with those edges along the axon, as remyelination proceeds, until reaching their ultimate position, where they are still bordered by Schwann cell edges. In some cases, two neighboring aggregates in the same axon appear to be "pushed" toward each other by the growing edges of the adjacent internodal segments and eventually fuse to form a single node between the two internodes (Dugandzija-Novakovic et al., 1995).

Whether or not nodal-type junctions or cytoskeletal interactions play a role in the *formation* of nodes, they may play a significant role in the *maintenance* of nodes, once formed, in the same way that extracellular elements are thought to anchor transmembrane receptors at synapses (McMahan, 1990). In the latter case, interaction with agrin is thought to result in phosphorylation of the receptors and aggregation. A similar mechanism could operate at nodes of Ranvier, in view of the presence of agrin there (Reist et al., 1987), although recent attempts to induce sodium channel aggregation by agrin in cultured myotubes have not succeeded (Lupa et al., 1991).

Steric hinderence due to closely applied paranodal junctions could also maintain nodal differentiation, while still allowing for turnover of nodal membrane components. A continuous helical line of axonal membrane winds through the paranodal region between the junctions formed by the glial loops. Node-like particles are visible within this linear path, as well as aggregates of them in "lakes" where the glial loops are not directly adjacent to each other (Figure 41-12). Particles within this helical channel are evidently not sterically hindered and could be en route, within the plane of the bilayer, to or from the nodal axolemma.

FUNCTIONAL SIGNIFICANCE OF AXOGLIAL INTERACTION IN NODAL AND PARANODAL REGIONS

Unlike the paranodal junctional regions, the nodal axolemma reveals no evidence of a paracrystalline pattern and contains, instead, large numbers of large particles, primarily in the E fracture face, where they are sharply demarcated on both sides by the paran-

odal junctions (Figures 41-5 and 41-12). The overall particle concentration in the E fracture face of the nodal axolemma is ~1000 to 1500 particles/μm^2 in both cold-blooded vertebrates (Rosenbluth, 1976; Tao-Cheng and Rosenbluth, 1980) and mammals (Kristol et al., 1978). The nodal channel density is at least an order of magnitude greater than that of the internodal axolemma, except in the juxtaparanodal region (Tao-Cheng and Rosenbluth, 1984) (Figure 41-12).

The characteristic nodal particles have attracted considerable attention, since they correspond to the voltage-gated sodium channels, known to be concentrated at the node, with respect to location, size, and concentration, and it has been hypothesized (Rosenbluth, 1976, 1981) that each particle represents one sodium channel, a glycoprotein of high molecular weight. This proposal has been further supported by freeze-fracture studies of purified sodium channels in artificial membranes (Ellisman et al., 1983), which show particles comparable in size and appearance to those at normal nodes of Ranvier. Thus, the distribution of the particles may correspond to the distribution of sodium channels within the axolemma, including those in regions that cannot be readily detected by electrophysiological methods.

The hypothesis was originally questioned in view of saxitoxin-binding studies suggesting a nodal particle density tenfold higher than that of the particles (Ritchie and Rogart, 1977). However, the saxitoxin studies were undoubtedly measuring sodium channels not only in the nodal axolemma but also in Schwann cells (Shrager et al., 1985) and perhaps also in the internodal axolemma, which is now known to contain far more sodium channels than the node itself, albeit at much lower concentration (Grissmer, 1986; Chiu and Schwarz, 1987). Data from a variety of sources (e.g., Chiu, 1980) have now established that nodal sodium channel density corresponds closely to that originally predicted by the E-face particle density (Rosenbluth, 1976).

Further support has been obtained from studies of the electric organ in *Sternarchus*, which consists of modified nerve fibers containing both excitable and inexcitable nodes (Waxman et al., 1972). Here too, membrane particle concentration has been shown to be high at the excitable nodes but low at the inexcitable ones (Kristol et al., 1977). Clearly the nodal E-face particles cannot represent voltage-gated potassium channels, since the latter have the converse distribution; that is, they are more concentrated in the internodal axolemma than at the node of Ranvier (Chiu and Ritchie, 1980; Kocsis and Waxman, 1980; Sherratt et al., 1980).

Thus, these membrane particles may represent sodium channels but are unlikely to represent potassium channels. On this basis, their distribution may be useful in inferring the electrical characteristics of axonal membranes under experimental or pathological conditions. For example, studies of nerve fibers from diabetic animals show no significant change in nodal particle density, suggesting no diminution in nodal sodium channels, but point, rather, to paranodal abnormalities as a basis for conduction slowing (Shirasaki and Rosenbluth, 1991). Conversely, the marked increase in axonal particle density in the axons of myelin-deficient rats probably reflects an abnormally high concentration of axonal sodium channels that underlies the spontaneous activity seen in these animals (Rosenbluth, 1990).

PATHOLOGY: EFFECTS OF MYELIN ABNORMALITIES ON AXOLEMMAL STRUCTURE AND FUNCTION

An extensive series of studies has produced clear evidence that the concentration and distribution of particles within the axolemma is markedly affected by the investing myelin sheath (Rosenbluth, 1981, 1988), and numerous studies have shown consistently that the distinctive pattern illustrated in Figure 41-12 occurs only in the axolemma of fibers invested by normal myelin sheaths.

In cases where myelin of aberrant form is laid down, some semblance of the normal nodal and paranodal membrane structure may be seen, the distortions in axolemmal particle distribution corresponding to the aberrations in the form of the overlying sheath. In cases where myelin is absent, the typical cylindrical, sharply demarcated axolemmal membrane specializations are also absent, although aberrant noncircumferential specializations may occur in association with glial processes.

For example, some differentiation of the axolemma has been shown in thin sections of demyelinated axons associated with astrocyte processes (Blakemore and Smith, 1983), and in freeze-fracture replicas of the periphery of ethidium bromide lesions, it has also been possible to find node-like particle accumulations corresponding to such axon-astrocyte junctions (Rosenbluth et al., 1985). It is clear, however, that such particle accumulations, in the absence of normal myelin sheaths, never develop the typical sharply defined 1μm cylindrical form shown in Figure 41-12. Rather, the particle patches tend to appear in elongated form and do not completely encircle the axon; that is, the nodal domain is not clearly separated from the internodal domain. Thus, contact with astrocyte processes

alone is associated with only partial differentiation of the axolemma. The fully differentiated pattern requires that the axon be normally myelinated as well. Although aberrant nodal and paranodal junctions occur at the periphery of the lesions, where astrocyte and oligodendrocyte processes are present, they do not occur in the core of the lesion, where glial processes are absent.

Similarly, in the peripheral nervous system, the amyelinated bundles that occur in the spinal roots of the dystrophic mouse mutant display aberrant nodal (Figure 41-5) and paranodal axolemmal specializations only at the periphery, where limited contact with Schwann cells occurs, but not in the glia-free interior of the bundles (Rosenbluth, 1979). Thus, in the absence of all glial contact, the axolemma shows no significant focal particle accumulations.

These studies indicate that differentiation of the axon depends on contact with glial elements—the myelin sheath and the perinodal processes. Once myelin has formed, however, and the axon has already become differentiated, does it retain that differentiated structure even if the investing myelin sheath is lost or altered? Several experimental studies bear on this question.

In the ethidium bromide model of demyelination, referred to above, the toxin affects all exposed cell bodies, including both oligodendrocytes and astrocytes, but not the axons passing through the lesion, whose cell bodies are remote. The glia and the myelin sheaths attached to them disintegrate, resulting in a focal glia-free region, which remains stable for many weeks. As described above, aberrant axoglial junctions occur at the periphery of such lesions, but examination of the core shows an abundance of apparently normal axons closely applied to each other with no evidence, thus far, of any nodal or paranodal axolemmal specializations. Since the spinal cord was fully myelinated at the time of the injection, this finding implies that whatever differentiated structures were associated with the original nodal and paranodal complexes must have disappeared; that is, the axons have dedifferentiated in this region in the absence of continuing glial contact. Equivalent results have been seen in the peripheral nervous system after antibody-mediated demyelination (Rosenbluth, 1981).

Thus, contact with glial cells appears necessary not only for the initial *development* of the differentiated form of the axolemma, but also for long-term *maintenance* of the differentiated structure of the axolemma. Accordingly, although the pre-existing axolemmal sodium channel distribution may persist temporarily following demyelination, it can be expected to change eventually, as the axon dedifferentiates, or to reorganize if reensheathement occurs.

In addition to its effect on the regional differentiation of the axolemma and sodium channel distribution, interaction with myelin-forming cells also appears to affect the overall number of axolemmal sodium channels. Particle counts suggest that the sodium channel density in the normal internodal axolemma is at least an order of magnitude lower than that in the nodal axolemma (Tao-Cheng and Rosenbluth, 1984). Nevertheless, the total surface area of the internode is so large that the total number of internodal channels is probably at least an order of magnitude greater than that at the node.

In a normal fiber, the internodal channels are presumably inactive, and only the nodal channels are involved in ion exchange. Nerve fibers that lack myelin sheaths, however, such as those seen in the myelin-deficient rat spinal cord, in some cases develop much larger concentrations of node-like membrane particles than are ever seen in normal internodes, suggesting higher-than-normal sodium channel densities (Rosenbluth, 1990). These regions, which may be extensive, appear in animals more than 3 weeks old, in which generalized seizures are manifest, but not in younger animals, before the development of seizures (Rosenbluth, 1990).

Recent saxitoxin-binding studies of the shiverer mouse mutant came to the same conclusion, that is, that the dysmyelinated fibers in that mutant also develop abnormally high sodium channel densities (Noebels et al., 1991). This effect was attributed specifically to myelin basic protein deficiency, though it is more likely to be a feature of myelin deficiency from any cause.

Thus, the presence of myelin appears to govern not only the distribution of sodium channels, but also their overall density. Specifically, myelination of an axon segment appears to downregulate insertion of sodium channels into the internodal axolemma of that segment.

Elimination of this regulatory factor might be beneficial or detrimental. If, in the case of a demyelinated axon, the sodium channel concentration in the bare, formerly internodal, region of an axon were to rise gradually, continuous conduction might be restored to an otherwise blocked fiber. Although reversal of conduction block by this means is desirable, and may underlie much of the functional return after acute attacks of multiple sclerosis, continuing increase of axolemmal sodium channel concentration could result in spontaneous activity due either to direct activation of low-threshold fibers by action currents generated in neighboring fibers or by spontaneous depolarization due to potassium accumula-

tion in the narrow interstitial spaces. These mechanisms may underlie the paroxysmal activity often seen in multiple sclerosis or in peripheral demyelinating conditions.

It is of interest too that the initial segment of spinal ganglion cells, which is ensheathed but not myelinated, also displays a much higher density of node-like membrane particles than that seen in the internodal axolemma of the same axon more distally (Matsumoto and Rosenbluth, 1985), suggesting that the same mechanism may regulate regional sodium channel density in normal fibers as well.

ASTROCYTES

Although the astrocyte is much less conspicuously divided into domains than the myelin-forming cells, its surface is nevertheless distinctly nonuniform with respect to the distribution of membrane components and junctions. In the case of astrocytes that form the glia limitans, the cell as a whole assumes a clearly polarized form that includes a region peripherally corresponding to the "base" of a classical epithelial cell.

Such astrocytes extend processes to the pia-glial junction where their apposed terminal expansions form both gap junctions and punctate adherent junctions. That portion of the cell facing the pia corresponds to the base of the cell in that it is covered by a basal lamina. Similarly, astrocytes extend processes toward capillaries that form incomplete sheaths around the vessels. Here too, that portion of each cell facing the capillary is covered by a basal lamina. Although goldfish astrocytes have been reported to form enormous networks of tight junctions and to be interconnected by desmosomes (Wolburg et al., 1983), neither of these specialized junctions has been seen at contacts between mammalian astrocytes (Mugnaini, 1986). Thus, the extent to which the membrane components of mammalian astrocyte domains remain segregated.

Another differentiated region of the cell consists of the microvillous processes that sometimes surround nodes of Ranvier forming the nodal axoglial junctions in the central nervous system corresponding to the junctions formed by the perinodal Schwann cell "fingers" in the peripheral nervous system. There is no basal lamina associated with the astrocyte microvilli, but an ill-defined extracellular matrix material occupies the perinodal extracellular space. In the peripheral nervous system, there is a clear separation between the perinodal matrix, which lies deep to the Schwann cell basal lamina, and the endoneurial extracellular space external to the basal lamina (Figure 41-12). The latter contains collagen and other obvious formed elements, while the perinodal matrix is amorphous. Histochemical studies suggest that the perinodal matrix is a proteoglycan, presumably secreted by the perinodal glial processes, which has ion exchange properties that serve to buffer the perinodal region with respect to sodium and potassium (Langley, 1979). In lower vertebrates, a similar matrix, distinct from the endoneurium, also surrounds the axon hillock and initial segment of spinal ganglion cells (Pannese, 1981; Matsumoto and Rosenbluth, 1986) and sympathetic ganglion cells (Taxi, 1976).

Astrocytes clearly form gap junctions (Figure 41-13) with each other and with oligodendrocytes as well (Mugnaini, 1986), thus coupling the macroglia into a functional syncytium capable of redistributing extracellular ions, especially potassium, and thereby maintaining the constancy of extracellular pH and ionic composition (Orkand et al., 1966). Many additional functions have been proposed for astrocytes, including isolation and sequestration of neurotransmitters and secretion of growth factors and other cytokines. The recently demonstrated calcium waves in astrocyte networks in response to exogenous glutamate or to nerve stimulation *in vivo* (Dani et al., 1992) may depend on gap junction coupling and may thus represent a signaling mechanism that serves to coordinate astrocyte behavior over broad areas. As discussed above, the passage of cAMP or other small molecules through gap junctions may subserve this purpose as well (see Chapter 21, this volume.)

The most intriguing astrocyte membrane specialization (Figure 41-13) consists of tetrads or orthogonal arrays of membrane particles known as *assemblies* (Dermietzel, 1974b; Landis and Reese, 1974). These occur also in the satellite cells of peripheral ganglia (Pannese, 1981) and have been found in muscle and other cells as well (for review see Landis, 1986). Although their morphology and distribution have been studied extensively, the function of these distinctive structures is still uncertain.

In view of the role that has been ascribed to astrocytes in regulating extracellular potassium and other ions, it has been proposed that the assemblies are involved in ion transport, perhaps representing channels that permit efflux of potassium taken up by astrocytes into pericapillary and subarachnoid regions, where the excess can be carried off by either the blood or the spinal fluid innocuously without altering the resting potential of neuronal processes. Of great interest is the fact that the concentration of assemblies on the astrocyte surface is greater in the pericapillary processes and at the pia-glial junctional

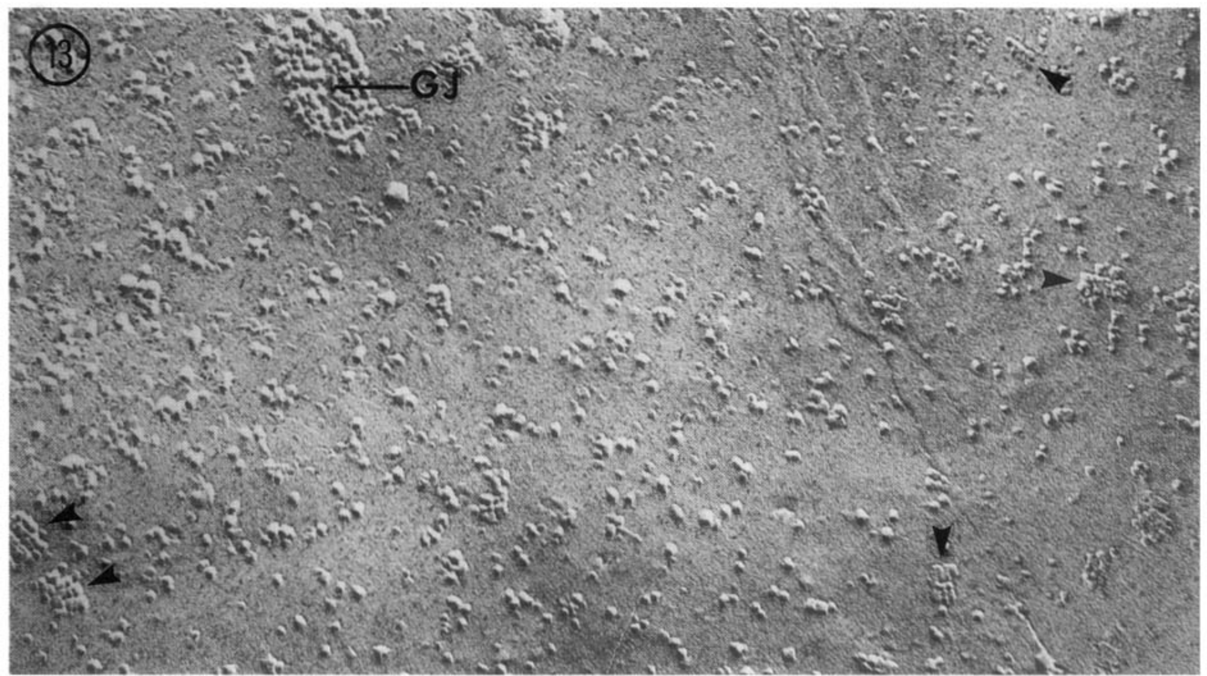

FIG. 41-13. Freeze-fractured astrocyte membrane (*P-face*) showing a patch of gap junction particles (*GJ*) and scattered rectangular "assemblies" (*arrows*).

endfeet than elsewhere, that is, precisely at the site where correlative physiological studies (Newman, 1986a) have shown potassium conductance to be tenfold higher than elsewhere along the astrocyte surface! Equivalent observations have been made on Müller cells, the counterparts of astrocytes in the retina (Newman, 1986b; Wolburg and Berg, 1988. See also Chapters 4 and 50).

The mechanisms that govern synthesis, formation, and distribution of the "assemblies" remain uncertain. Coculture of astrocytes with endothelial cells induces their appearance along the astrocyte membrane facing the endothelial cells (Tao-Cheng et al., 1990). Thus, in this case, it appears that the regional differentiation of the astrocyte membrane is under the influence of adjacent cells. The mechanism of this interaction is not known, however. Moreover, no one has succeeded in isolating assemblies in subcellular fractions or characterizing them biochemically. Nor are any mutations known in which these structures are absent. Surprisingly, although they are abundant in mammalian astrocytes, they have not been seen in amphibian or fish astrocytes; yet, they are present in the Müller cells of all three classes (Berg-von der Emde and Wolburg, 1989).

SUMMARY

Oligodendrocytes, astrocytes and Schwann cells are divided into subcellular domains that display distinctive surface structures as well as specialized junctions between the glial cells themselves and between glial cells and axons. The axoglial junctions are of two types, nodal and paranodal, both of which affect the structural differentiation of the axolemma and the distribution of ion channels within it. The relevance of these various structures to the behavior of both the glia and the axons, under normal and pathological conditions, is discussed.

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42 Cytoskeletal organization of myelinating Schwann cells

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In 1954, Geren demonstrated with the electron microscope that the myelin sheath is an elaborate specialization of the Schwann cell plasma membrane, which is spirally wrapped around and separate from the axon. Although our knowledge of the biochemistry, cell biology, and molecular biology of myelin and myelination has increased tremendously since then (see Chapters 38 and 39), many questions about basic mechanisms of myelination remain unanswered. Very little is known about how spiral growth of the myelin internode occurs, how axons regulate myelination, or how myelin molecules are targeted to different sites millimeters away from their point of synthesis. Because the cytoskeleton is integral to membrane growth and intracellular transport, understanding the organization and specializations of the Schwann cell cytoskeleton will help elucidate many of the basic mechanisms of Schwann cell myelination.

The cytoskeleton consists of three morphologically and biochemically distinct networks: *microfilaments* (MFs), *microtubules* (MTs), and *intermediate filaments* (IFs). This chapter focuses on the distribution and organization of these networks and their potential functions during myelination. The cytoskeleton is a dynamic and versatile organelle system that is essential for many cellular functions. *Actin, tubulin,* and *vimentin* provide the structural framework for MFs, MTs, and IFs in myelinating Schwann cells, and their distributions have been described (Trapp et al., 1989; Zimmerman and Vogt, 1989; Kobayashi and Suzuki, 1990; Kordeli et al., 1990; Kidd et al., 1993; Trapp et al., 1993). Molecules that bind to F-actin, tubulin, or vimentin endow the cytoskeleton with specialized functions. The identification and distribution of these molecules in myelinating Schwann cells are largely unknown. It is tempting to speculate that Schwann cells express unique cytoskeletal molecules that have evolved to help mediate the membrane expansion and protein targeting that is required during myelination.

THE PERIPHERAL NERVOUS SYSTEM MYELIN INTERNODE

Before discussing the distribution and organization of the Schwann cell cytoskeleton, and its roles in myelination, it is important to understand the size and complexity of myelin internodes in the peripheral nervous system. Myelinating Schwann cells develop from small, relatively undifferentiated cells into myelin internodes that can be as much as 2 mm long and contain 5 mm of myelin spiral (Friede and Bischhausen, 1980). This tremendous expansion of the Schwann cell plasma membrane occurs within a few postnatal weeks and is a highly ordered process. The ultimate extent of myelin growth is determined by the axon.

As illustrated in Figure 42-1, the myelin internode is more than a simple extension of the Schwann cell plasma membrane. It contains several ultrastructurally and biochemically distinct membrane domains that include the outer plasma membrane of the Schwann cell, compact myelin, the Schmidt-Lanterman incisures, paranodal loops, nodal microvilli, the periaxonal membrane, and the membranes of the outer and inner mesaxons. These membranes contain different structural proteins (Figure 42-2). The P_0 glycoprotein (P_0), peripheral myelin protein 22, and myelin basic protein are enriched in compact myelin (Trapp et al., 1981; Omlin et al., 1982). The myelin-associated glycoprotein is concentrated in membranes of the paranodal loops, Schmidt-Lanterman incisures, periaxonal membranes, and inner and outer mesaxons (Figure 42-2) (Trapp et al., 1984). Laminin, an extracellular matrix component, is secreted from and attached to the Schwann cell plasma membrane (Cornbrooks et al., 1983). The subdivision of the myelin membranes requires elaborate pathways for protein transport and targeting. For instance, P_0, myelin-associated glycoprotein, and laminin are synthesized in the rough endoplasmic reticulum and Golgi membranes that are concentrated in the perinuclear cytoplasm. These proteins are sorted into different carrier vesicles as they exit the

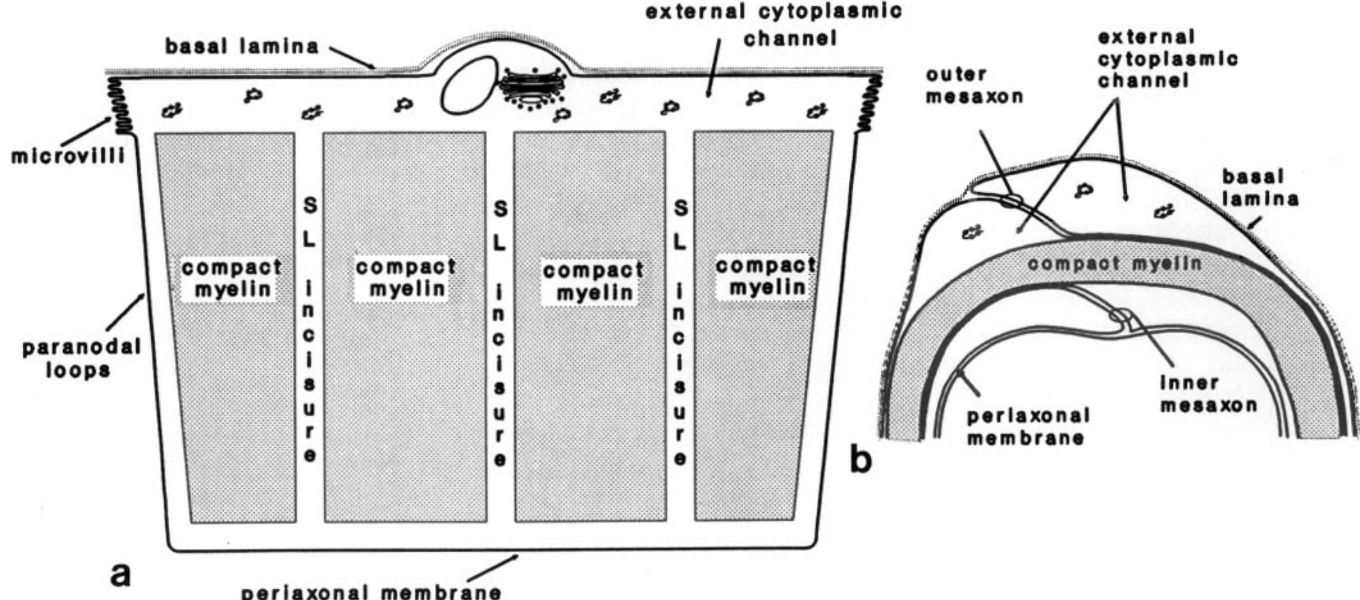

FIG. 42-1. Membrane domains and cytoplasmic compartments of peripheral nervous system myelin internodes. (A) A Schwann cell and its "unrolled" myelin internode. Most of the internode consists of compact myelin. Schmidt-Lanterman (*SL*) incisures are cytoplasmic channels that extend through regions of compact myelin. At either end of the myelin internode, the sheath terminates in cytoplasmic channels that form the paranodal loops. Microvilli extend from the Schwann cell to cover the nodal axolemma. (B) A partial cross section through a myelin sheath illustrates the outer and inner mesaxons. The Schwann cell plasma membrane is surrounded by a basal lamina.

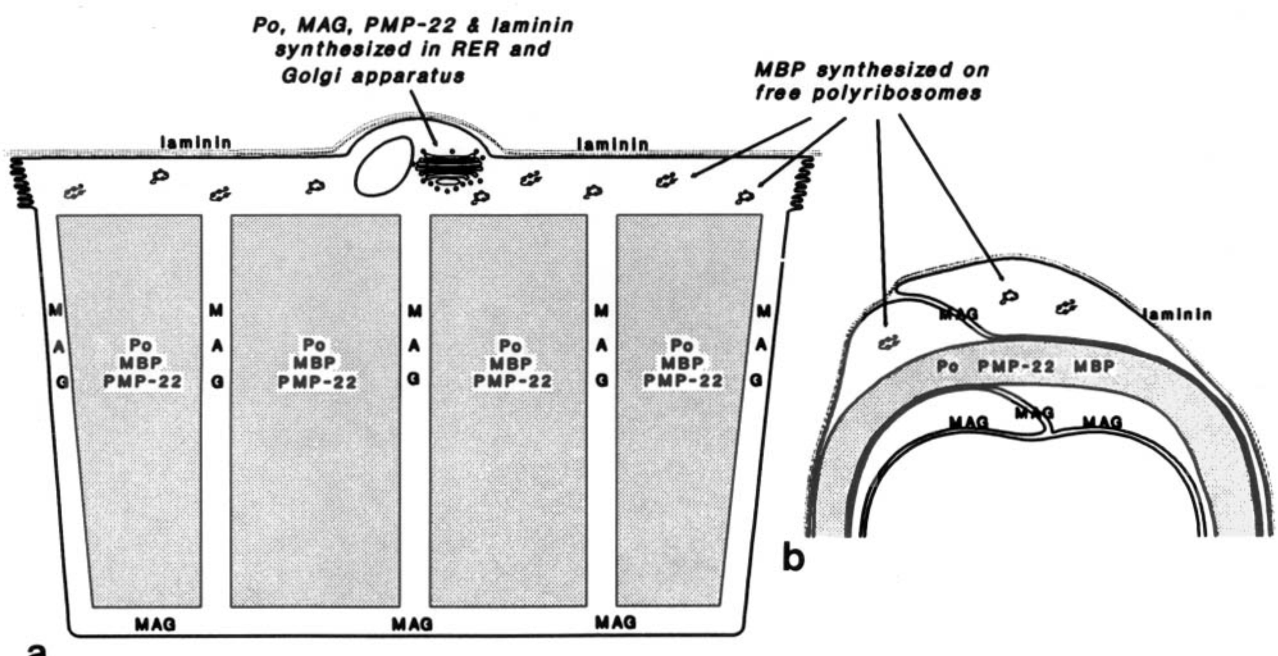

FIG. 42-2. Distribution and sites of synthesis of myelin proteins in (A) an "unrolled" myelin internode and (B) partial cross section. P_0, peripheral myelin protein 22 (PMP-22), and myelin basic protein (MBP) are enriched in compact myelin membranes (*stippled*). Myelin-associated glycoprotein (MAG) is enriched in membranes of the Schmidt-Lanterman incisures, paranodal loops, periaxonal region, and outer and inner mesaxons. The basal lamina component, laminin, is secreted from the outer (abaxonal) plasma membrane (see also Figure 42-4). P_0, PMP-22, MAG, and laminin are all glycoproteins that are synthesized in the perinuclear cytoplasm. MBP mRNA is transported within the external cytoplasmic channels and translated on free polyribosomes at sites along the myelin internode.

trans Golgi network (Trapp et al., 1993), transported over millimeter distances, and then inserted into different surface membranes. P_0 and myelin basic protein are both enriched in compact myelin but are transported very differently: P_0 by vesicular transport and myelin basic protein by translocation of its mRNA (Colman et al., 1982; Trapp et al., 1987; Griffiths et al., 1989). The cytoskeleton plays essential roles in membrane assembly, movement, and growth, and participates in protein and mRNA transport and the organization of synthetic organelles. It is not surprising that the cytoskeleton of myelinating Schwann cells is dynamic, complex, and modulated by axonal influences.

MICROFILAMENTS

MFs, which are the smallest of the cytoskeletal filaments (6 to 8 nm in diameter), consist of helical assemblies of actin monomers. They are polarized structures, in that they elongate by preferentially adding monomers to one end, termed the (+) or *barbed* end. Actin can be removed from MFs from the (−) or *pointed* end. Monomer addition is dependent on adenosine triphosphate (ATP) hydrolysis, and assembly and disassembly are dynamically regulated by a number of accessory proteins and by other factors, such as pH and ionic conditions (Pollard, 1990). Microfilament networks are organized by a large number of actin-binding molecules (see reviews by Carraway and Carraway, 1989; Heintzelman and Mooseker, 1992; Stossel, 1993) that promote or inhibit MF growth, cross-link MFs, sever MFs, or attach them to cell surface molecules and organelles. Elongation, contraction, and disassembly of MFs provide the force behind membrane movements that are required for cell growth and motility. Myosins serve as MF motors; they contract the MF network by sliding the filaments past one another and providing movement of membranes and membrane organelles on MFs.

There are two types of MF assemblies in nonmuscle cells. One is attached to the plasma membrane, forming the cell cortex, and is characterized by a nonparallel MF organization. Microfilaments may also be bundled into parallel arrays that form stress fibers and microvilli (Carraway and Carraway, 1989; Heintzelman and Mooseker, 1992). In myelinating Schwann cells, MFs are enriched beneath the membranes of the Schmidt-Lanterman incisures, the paranodal loops, the periaxonal membrane, the outer and inner mesaxons, and portions of the outermost compact myelin lamella and Schwann cell plasma membrane (Trapp et al., 1989; Zimmerman and Vogt, 1989; Kordeli et al., 1990) (Figures 42-3 and 42-4). These distributions were determined by phallicidin labeling of filamentous actin (Trapp et al., 1989; Zimmerman and Vogt, 1989). *Spectrin,* an MF cross-linking protein, and *ankyrin,* which binds spectrin to integral membrane proteins (Branton et al., 1981; Low, 1986; Carraway and Carraway, 1989), colocalize with filamentous actin in Schwann cells (Trapp et al., 1989). Together these three components form the framework for MFs beneath plasma membranes. The spatial arrangement of MFs in the cortex of the Schwann cell is unknown. In electron micrographs, the cortical MF network typically appears as a thin layer of largely amorphous material undercoating the Schwann cell plasma membrane (e.g., see Peters et al., 1991). This appearance may belie a highly organized submembranous MF network, however; in other cells, MFs form latticeworks beneath the plasma membrane that restrict movement of membrane-associated molecules and promote their interaction (see Edidin, 1992). The only consistently observed MF bundles in myelinating Schwann cells form the core of nodal microvilli (Landon and Williams, 1963; Raine, 1982); occasional accumulations of actin filaments have been observed in periaxonal cytoplasm (Jacobs and Cavanagh, 1972), but their organization and functional significance are unclear.

Functions of Microfilaments in Myelinating Schwann Cells

Membrane Movement. A phenotypic hallmark of myelinating Schwann cells is membrane expansion and membrane movement. During formation of the myelin internode, the periaxonal membrane ensheathes the axon and the outer or inner mesaxons or both must rotate to increase the number of myelin lamellae. Schmidt-Lanterman incisures are believed to migrate along the myelin sheath (Mugnaini et al., 1977), and paranodal loops retract to internodal locations in response to axonal swelling (Griffin et al., 1987). Because MFs are concentrated beneath all of these membranes (Figure 42-4), and because myosin is present in all cytoplasmic compartments of the myelin internode (Unsicker et al., 1978; Zimmerman and Vogt, 1989), it is reasonable to suggest that MFs drive membrane movement in myelinating Schwann cells as they do in other cells (see Stossel, 1993).

For membrane movement to occur, MFs must be attached to a membrane components. Because rotational growth is a unique feature of myelination, it is likely to involve a molecule specific to myelin-forming cells. It has been proposed that an interaction between myelin-associated glycoprotein (MAG)

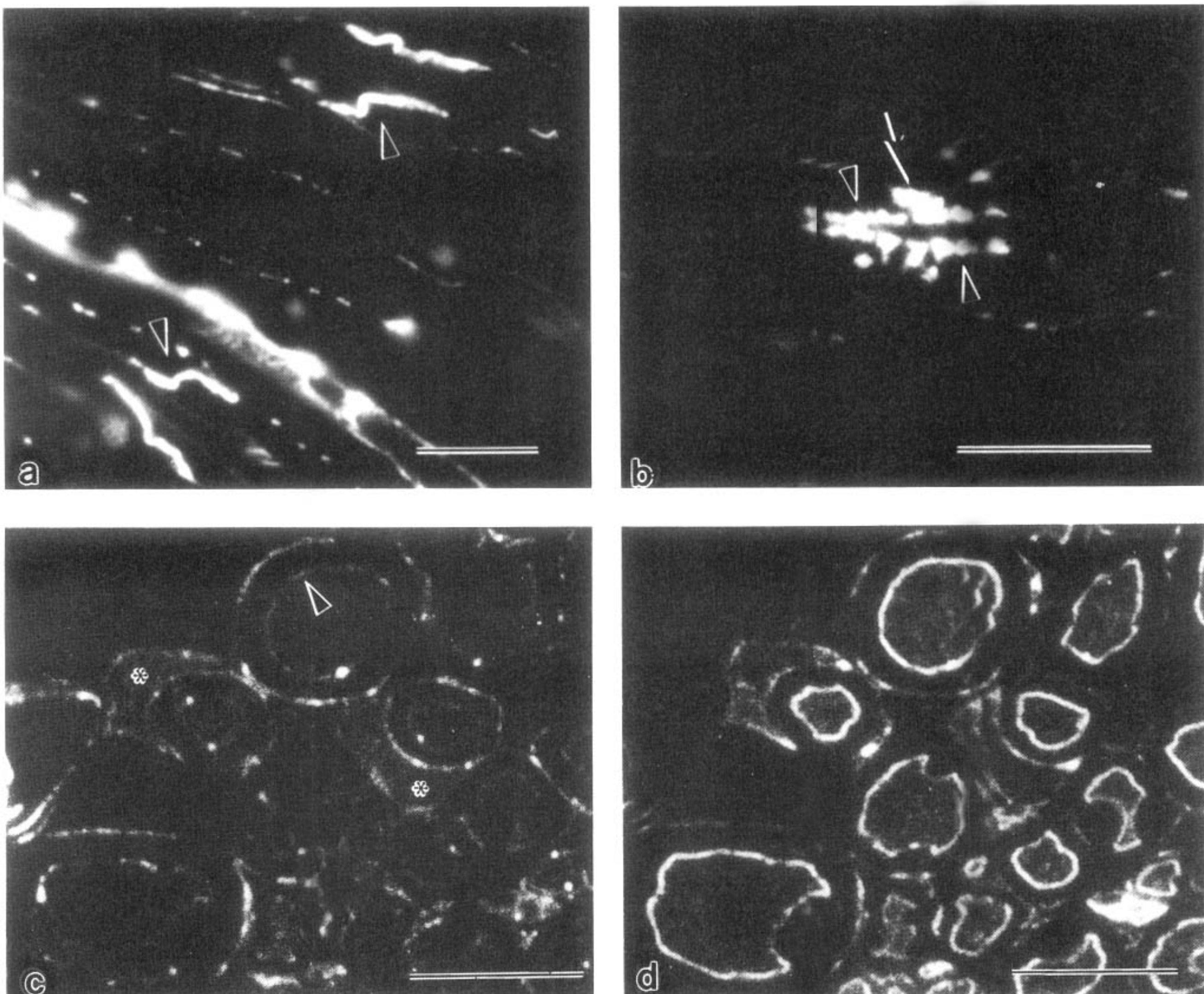

FIG. 42-3. Cryosections of adult rat sciatic nerve (1 μm): (A,B) stained for F-actin (rhodamine-phallicidin), and (C,D) double-labeled for F-actin and spectrin. F-actin is enriched in Schmidt-Lanterman incisures (Figure A, *arrowheads*) and also within the paranodal loops (Figure B, *arrowheads*) and nodal microvilli (*arrow*). In transverse sections, F-actin is present beneath periaxonal (Figure C, *arrowhead*) and abaxonal plasma membrane, and also adjacent to the outer compact lamella (*asterisks* denote external cytoplasmic channels). Spectrin (Figure D) colocalizes with F-actin at all locations, although staining intensities differ. Scale bars = 10 μm. [From Trapp et al. (1989), with permission.]

and MFs may play a role in membrane motility during myelination (Trapp and Quarles, 1982; Trapp et al., 1984; Martini and Schachner, 1986; Salzer et al., 1987). MAG is a member of the immunoglobulin gene superfamily and consists of a large extracellular domain that contains six IgG-like regions, a single transmembrane domain, and a cytoplasmic domain (Arquint et al., 1987; Lai et al., 1987; Salzer et al., 1987). MAG colocalizes with MFs (Figure 42-3) at membranes that move during internodal growth (Trapp et al., 1989). When MAG-containing membranes move, they use another membrane as a substrate and appose this membrane by a 12- to 14-nm gap. A number of studies indicate that MAG is responsible for maintaining this gap by acting as an adhesion molecule or a membrane spacer (Trapp and Quarles, 1982; Trapp et al., 1984; Martini and Schachner, 1986; Salzer et al., 1987). Transfection of additional copies of MAG into myelinating Schwann cells induces precocious spiral wrapping (Owens et al., 1990), whereas spiral wrapping is impaired or completely prevented in Schwann cells in which MAG expression is reduced or eliminated (Owens and Bunge, 1991). MAG, therefore, is a prime candidate for mediating movement of membranes in myelinating Schwann cells, either as part

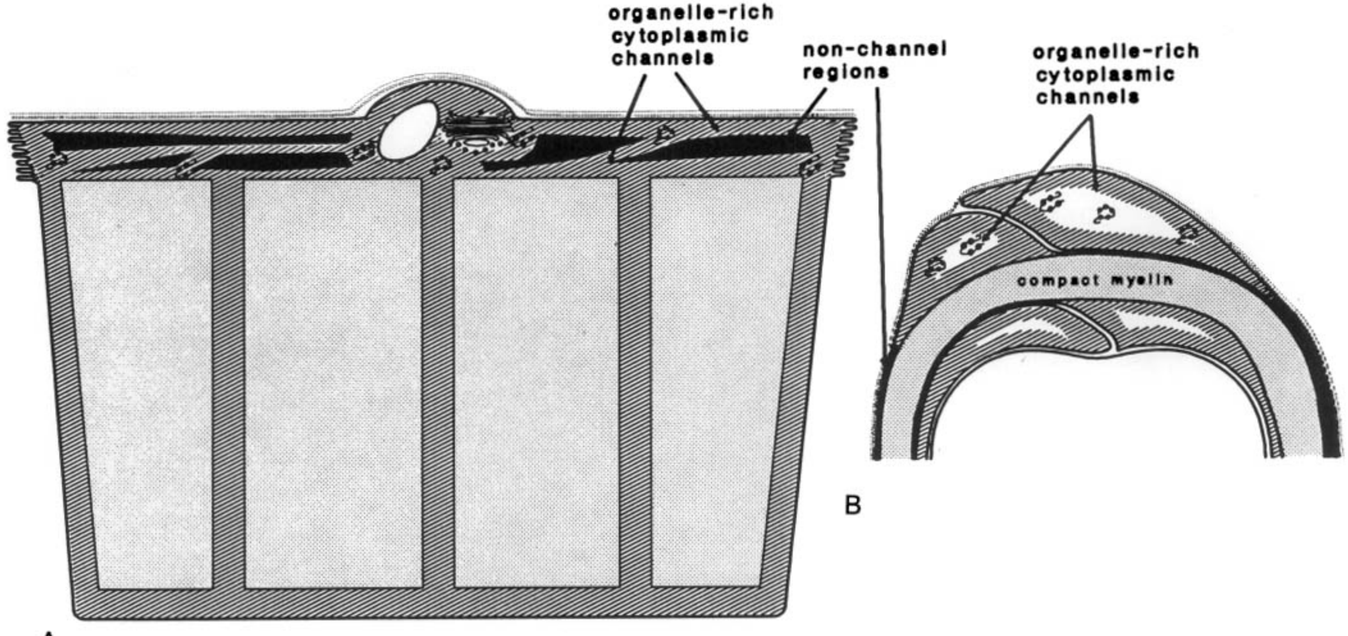

FIG. 42-4. Distribution of microfilaments in peripheral nervous system myelin internodes in (A) an "unrolled" internode and (B) a partial cross section. Microfilaments (*cross-hatching*) are enriched beneath the periaxonal membrane and the membranes of Schmidt-Lanterman incisures, paranodal loops, outer and inner mesaxons, and nodal microvilli. The cytoplasm at the outer margins of the myelin internode is normally subdivided into organelle-rich channel regions that are demarcated by microfilaments and nonchannel regions (*black*) that are devoid of organelles or detectable submembranous microfilaments.

of the motor attached to MFs or as an anchor that permits MF-mediated movement. A dynamic interaction between MFs and the cytoplasmic domain of MAG could explain how MAG-containing membranes are associated with stable membrane interactions in mature sheaths and with membrane movement during myelin formation.

Maintaining Cytoplasmic Channels. The size and geometry of the myelin internode necessitates an elaborate network of cytoplasmic channels that surround and traverse compact myelin (Mugnaini et al., 1977; Peters et al., 1991). These channels, which contain organelles, are important to formation and maintenance of the myelin internode and to intracellular transport of myelin components. They include the external cytoplasmic channels, Schmidt-Lanterman incisures, paranodal loops, and periaxonal cytoplasm (Figures 42-1 and 42-4). Microfilaments are associated with the membranes that demarcate these channels. The outer perimeter of the myelin internode is divided into organelle-rich channel regions and organelle-free nonchannel regions (Figure 42-4). Microfilaments are concentrated beneath the membranes of the organelle-rich channel regions. Because MFs demarcate the organelle-rich cytoplasmic domains of the myelin internode, it is reasonable to suggest that they help maintain these domains.

Membrane Interaction with Axons and Extracellular Environment. Similar to polarized epithelial cells, myelinating Schwann cells have two major surfaces. The abaxonal plasma membrane apposes the external endoneurial environment and the adaxonal periaxonal membrane apposes the axon. The microfilaments are enriched beneath both of these membranes (Trapp et al., 1989; Zimmerman and Vogt, 1989). Microfilaments associated with these plasma membranes are likely to have multiple functions dealing with the external environment. Microfilaments participate in endocytosis (Blok et al., 1982), pinocytosis (Phaire-Washington et al., 1980), and exocytosis (John et al., 1983; Koffer et al., 1990) and may mediate or regulate processes such as endocytosis of cholesterol (Boyles et al., 1989; Rothe and Müller, 1991) and exocytosis of extracellular matrix components (Cornbrooks et al., 1983). Cortical MFs can also provide resistance to externally applied stresses and transmit them to other cytoskeletal components (Wang et al., 1993). Stress resistance may be particularly important in Schwann cells, which are frequently subjected to compressive and traumatic events.

Several transmembrane molecules may anchor and organize the MF network beneath the Schwann cell plasma membrane. The abaxonal membrane is attached to a basal lamina, the formation of which

is a prerequisite for Schwann cell myelination to occur (Bunge et al., 1990). Schwann cells interact with the basal lamina through integrins, a family of transmembrane glycoproteins with a cytoplasmic domain that can be anchored to MFs by actin-binding proteins (Buck and Horwitz, 1987; Hynes, 1987; Wang et al., 1993). The α2-β1 integrin isoform has been identified on the surface of myelinating Schwann cells from human nerves (Hsiao et al., 1991). Schwann cells may also interact with the basal lamina through heparan sulfate-containing proteoglycans, which bind to extracellular matrix components (Hynes and Yamada, 1982). One membrane-spanning proteoglycan (Mehta et al., 1985; Carey and Todd, 1986) is exposed on the Schwann cell surface and is bound to an actin/spectrin/vimentin-rich cytoskeleton (Carey and Todd, 1986). The basal lamina surrounds the entire external surface of myelinating Schwann cells, but MFs appear to be enriched beneath the plasma membrane only where cytoplasmic channels are present. This raises two possibilities: integrins and other extracellular matrix receptors in the nonchannel regions are not attached to MFs, or MFs are present in the nonchannel regions at nondetectable concentrations. At the adaxonal surface of myelinating Schwann cells, MFs may be attached to adhesion molecules such as MAG (Trapp et al., 1989; Trapp, 1990).

Paranodal Membrane Specialization. The main function of myelin is to insulate the axons so that propagation of the action potential can occur by saltatory conduction (Funch and Faber, 1984). Current flow or ion exchange associated with the action potential does not occur through axolemma covered by myelin. Instead, current flow occurs at nodes of Ranvier, where the axolemma contains a high concentration of voltage-sensitive sodium channels. The paranodal loops and nodal microvilli are believed to serve an important role in buffering and maintaining the nodal environment (Landon and Williams, 1963; Thomas et al., 1993). Microfilaments form the structural core of the microvilli (Raine, 1982), which extend from the outer paranodal loops to surround the nodal axolemma. Microvilli increase the absorption/secretion surface area of membranes at nodes and therefore also the buffering power of these membranes (Thomas et al., 1993).

An isoform of ankyrin, ankyrin R, is selectively enriched in paranodal regions of peripheral nervous system myelin internodes (Kordeli et al., 1990). Microvilli and paranodal membranes also contain Na^+/K^+ ATPases (Ariyasu and Ellisman, 1987) and sodium channels (Ritchie et al., 1990). Ankyrin R can bind to both molecules (Koob et al., 1987; Nelson and Veshnock, 1987; Srinivasan et al., 1988), raising the possibility that ankyrin R plays a role in stabilizing and/or targeting sodium channels and Na^+/K^+ ATPases to specific regions of the myelin internode. These studies have provided the first evidence for selective enrichment of MF components in specialized regions of myelinating Schwann cells.

Myelin Protein Insertion. Another Schwann cell membrane in which MFs may have special functions is the outer compact myelin lamella in regions of the organelle-rich cytoplasmic channels. This membrane is unique in that its extracellular leaflet forms an intraperiod line with the underlying compact myelin sheath, while its cytoplasmic leaflet is associated with MFs. Autoradiographic studies implicate this membrane as the principal site for addition of myelin proteins during growth of the myelin sheath (Gould, 1977). Addition of P_0 to compact myelin most likely occurs by fusion of carrier vesicles, and may involve a specialized fusion receptor (Trapp et al., 1993). Microfilaments could potentially aid in this fusion or exert control over fusion sites by determining the distribution and/or properties of putative receptors. Microfilaments associated with this outermost compact myelin lamella may also contribute to myelin basic protein (MBP) synthesis and the targeting of MBP to compact myelin. Myelin basic protein is a heavily charged molecule that binds nonspecifically to many lipids (Smith, 1977; Boggs and Moscarello, 1978). Translation of MBP close to the site of incorporation into myelin assures its association with myelin and prevents binding to other organelles. Microfilaments participate in localization of mRNA and ribosomes in other cells (Singer et al., 1989; Yisraeli et al., 1990; Sundell and Singer, 1991), and may bind and stabilize MBP mRNA in the Schwann cell periphery. Microfilaments associated with the outer compact myelin lamella may selectively bind MBP mRNA-containing polyribosomes and thus ensure immediate association of newly synthesized MBP with compact myelin.

MICROTUBULES

MTs, the largest of the cytoskeletal filaments, provide dynamic substrates for the trafficking and structural organization of most organelles (for reviews, see Dustin, 1984; Kirschner and Mitchison, 1986; Schroer and Sheetz, 1991). Microtubules are cylindrical filaments that have an external diameter of about 25 nm. They are constructed of heterodimers of α and β tubulins, which polymerize in a guanosine triphosphate (GTP)-dependent process into fila-

ments that may reach 700 μm in length (Tsukita and Ishikawa, 1981). Microtubule growth is asymmetric; subunits are preferentially added and removed at the (+) or "fast-growing" end. Subunit addition at the (−) end is slow, and *in vivo*, the (−) end is frequently bound to an MT-nucleating structure, such as the centrosome. Microtubule asymmetry is important to MT-based transport, because the MT motors that transport organelles move preferentially toward either the (+) or (−) end of the MT. The factors that regulate motor direction, motor activity, and motor interactions with organelles remain poorly understood.

The size and complex geometry of the peripheral nervous system myelin internode (Figure 42-1) requires special mechanisms for synthesis, sorting, site-specific targeting, and stabilization of myelin proteins to the appropriate membrane domains (Figure 42-2). Microtubules have a well-established role in intracellular transport (Dustin, 1984; Schroer and Sheetz, 1991) and specialized MT configurations can mediate site-specific targeting (Hugon et al., 1987; Achler et al., 1989; Bre et al., 1990; Gilbert et al., 1991). In myelinating Schwann cells, the distribution and organization of MTs (Kidd et al., 1993) and studies of MT disruption (Hansson and Sjostrand, 1971; Jacobs et al., 1972; Roytta et al., 1984; Trapp et al., 1993) demonstrate that MTs are crucial to transport of myelin proteins and organelles. The transport and structural functions of MTs are governed by their orientation and organization, which in Schwann cells are influenced by axons (Kidd et al., 1993). Although the precise molecular interactions involved in transport of myelin proteins remain uncertain, MT disruption studies have provided insight into mechanisms mediating sorting and transport of myelin proteins (Trapp et al., 1993).

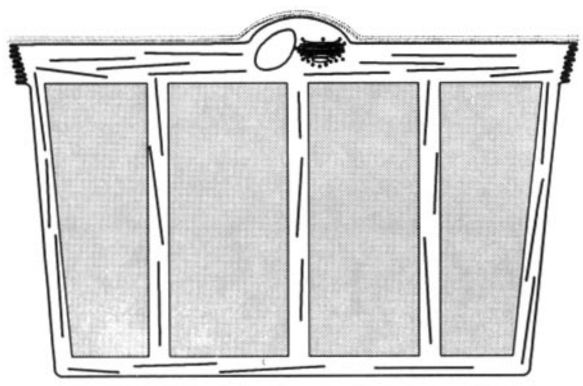

FIG. 42-5. In myelinating Schwann cells, microtubules are numerous in the outer cytoplasmic channels and perinuclear cytoplasm. Fewer microtubules are present in Schmidt-Lanterman incisures, paranodal loops, and periaxonal cytoplasm.

Distribution and Organization of Microtubules

MTs are present in all major cytoplasmic compartments of the myelin internode, but are excluded from compact myelin (Figure 42-5) (Peters et al., 1991). Thus, MTs have the potential to transport vesicles and organelles within all major cytoplasmic regions of the internode. They are numerous in the perinuclear region and in the cytoplasmic channels that lie external to the outer margin of the compact sheath (Peters et al., 1991). Microtubules are also present in Schmidt-Lanterman incisures, paranodal loops, and the periaxonal cytoplasm (Peters et al., 1991).

The organization of MTs is determined in part by their site of nucleation, which fixes, at least initially, the location of the MT (−) end. In many cells such as fibroblast, MTs are nucleated from a filamentous material concentrated around the centrosome (Dustin, 1984), forming an MT-organizing center. Myelinating Schwann cells have a more complex arrangement in which MTs extend from many sites scattered throughout the perinuclear cytoplasm and not from the centrosome (Figure 42-6) (Kidd et al., 1993). Microtubule-nucleating sites have not been detected at other internodal locations. Thus, there are no MT-organizing centers along the internode that may have served as specific (−)-end transport sites, analogous to the apical surface of epithelial cells (Hugon et al., 1987; Achler et al., 1989; Bre et al., 1990; Gilbert et al., 1991).

Although MT-nucleating sites are restricted to perinuclear cytoplasm, MT polarity in the external cytoplasmic channels is mixed (Figure 42-7) (Kidd et al., 1993), providing an MT network in which both (+)-end-directed and (−)-end-directed transport may move organelles from the perinuclear region. About 75% of MTs have their (−) ends toward the nucleus (Figure 42-7), whereas 25% have (−) ends away from the nucleus; this (+)-end to (−)-end ratio is maintained along the internode. Myelinating Schwann cells, therefore, are probably nucleated in the perinuclear cytoplasm, then detached from these nucleating sites, and transported along the internode (Figure 42-8). A similar mechanism has been proposed for axonal MTs (Hoffman and Lasek, 1975; Black and Lasek, 1980; Baas and Ahmad, 1992). Microtubules with their (−) ends directed away from the perinuclear region could be generated in two ways. First, some may be transported from the perinuclear cytoplasm in that orientation, for instance, by (−)-end-directed MT sliding, as occurs in flagella (Dustin, 1984). Alternatively, all MTs may

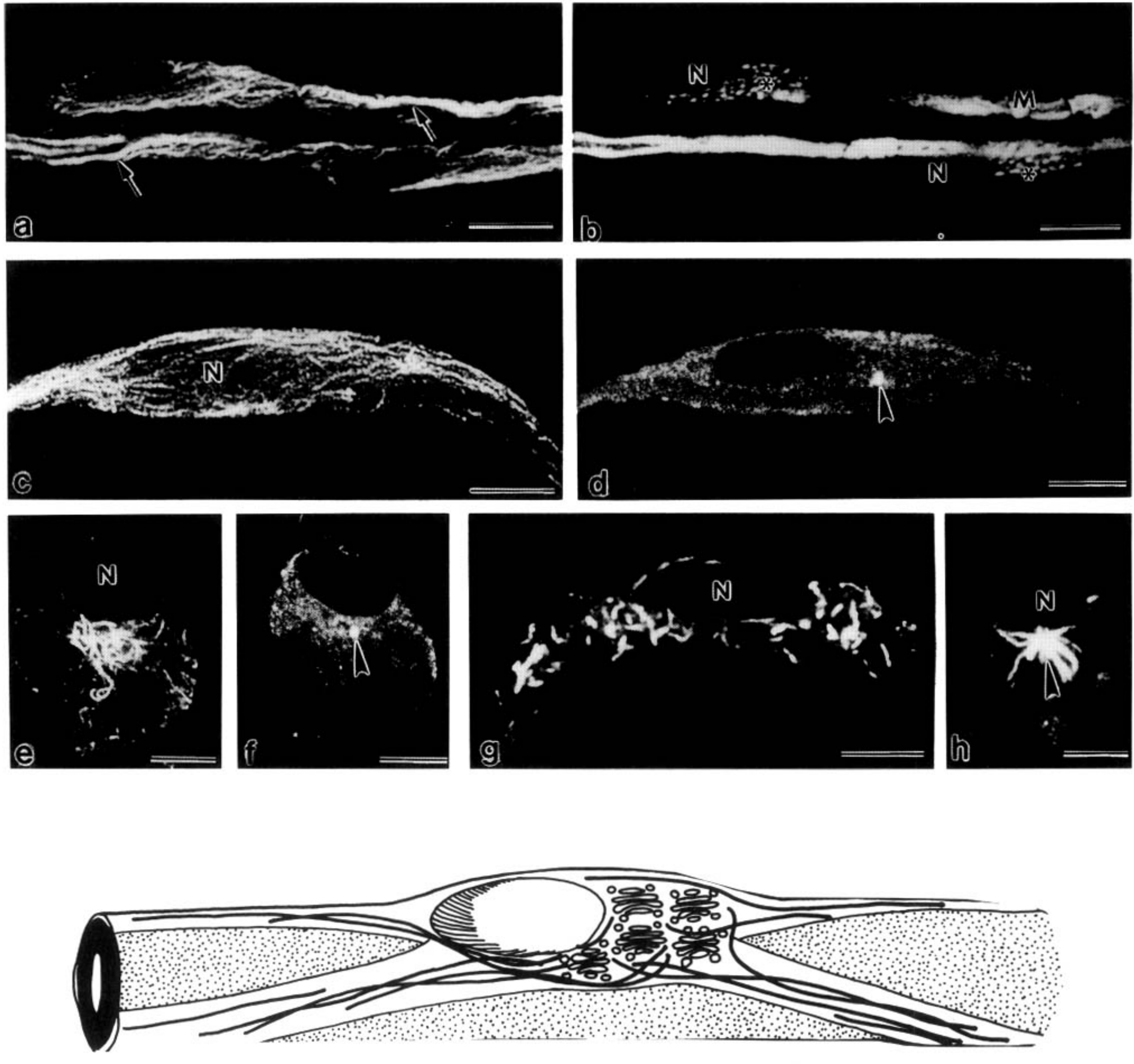

FIG. 42-6. Confocal fluorescence micrographs of myelinating Schwann cells (*a–d, g*) and perineurial cells (*e, f, h*) from rat sciatic nerve after immunostaining for microtubules (acetylated α-tubulin; *a–c, d, g, h*), the Golgi apparatus (P_0 antibody; *b*), and the centrosome (pericentrin; *d, f*). In developing internodes (*a, b*) microtubules are concentrated in the perinuclear region and extend into the external cytoplasmic channels (*arrows*). There is no discrete microtubule-organizing center (*c, d*), and Golgi profiles (*) are scattered throughout the cytoplasm at one longitudinal end of the nucleus (*N*). In contrast, perineurial cells from the same tissue (*e, f*) have a prominent centrosomal microtubule-organizing center. After colchicine-induced microtubule depolymerization, microtubules repolymerize at numerous sites within the perinuclear region (*g*), and not at a single aster, unlike fibroblasts (*h, arrow*). The organization of microtubules is summarized in (i). Scale bars = 10 μm. [From Kidd et al. (1993), with permission.]

initially be transported with (−) ends toward the perinuclear region (Figure 42-8), as proposed in axons (Baas and Ahmad, 1992). Later, the normal cycles of catastrophic depolymerization and repolymerization reduce MT length and permit 180 degrees rotation. Microtubules subsequently elongate with (+) ends pointed toward the perinuclear region. In either scenario, other factors must limit (−)-end-directed transport or MT rotation to only 25% of the total MT population.

In addition to MT nucleation sites and MT transport, stabilization can also modulate MT function.

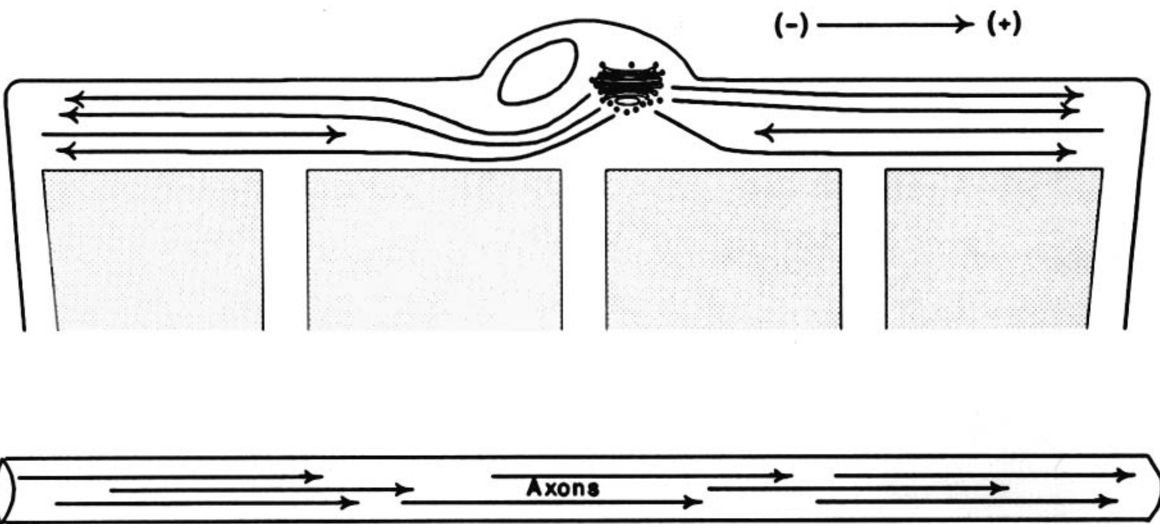

FIG. 42-7. Microtubule polarity in myelinating Schwann cells was determined by using the hook method. Seventy-five percent of Schwann cell microtubules had (+) ends away from the perinuclear region, whereas the remainder had the opposite polarity. In contrast, 95% of axonal microtubules had a uniform polarity.

Microtubules are inherently unstable and oscillate between phases of elongation and collapse (Dustin, 1984; Kirschner and Mitchison, 1986). The extent of MT depolymerization and repolymerization is determined by complex assembly/disassembly kinetics and can be influenced by modifications such as binding of MT-associated proteins (MAPs) (Sloboda et al., 1976; Pryer et al., 1992). Myelinating Schwann cell MTs are enriched in acetylated α-tubulin epitopes (Kidd et al., 1993), a posttranslational modification characteristic of MTs with long half-lives (Cambray-Deakin and Burgoyne, 1987; Piperno et al., 1987). Myelinating Schwann cells also contain MAP1a, MAP1b, and tau (Kidd et al., 1993), which could participate in stabilization of MTs. Stabilization increases MT density without the metabolic burden of producing and transporting many more labile MTs, and is particularly important in cells with long processes, such as Schwann cells and axons.

Microtubule Functions in Myelinating Schwann Cells

Protein Transport. Microtubules provide a substrate for rapid transport of organelles, which is mediated by molecular motors in an energy-dependent manner. These motors bind to both the MT and the organelle being transported, and different motors preferentially translocate material toward either the (+) or (−) end of MTs (Vale et al., 1985; Paschal and Vallee, 1987; Paschal et al., 1987; Schroer et al., 1989). The involvement of MTs in transporting and targeting P_0 protein, MAG, and laminin to different surface membranes has been investigated (Trapp et al., 1993). All three proteins are synthesized in rough endoplasmic reticulum and Golgi and transported to surface membranes as part of membrane carrier vesicles that bud from the *trans*-Golgi network. Microtubule disassembly causes marked accumulation of P_0, MAG, and laminin in the perinuclear cytoplasm of myelinating Schwann cells (Trapp et al., 1993) (Figure 42-9), indicating that MTs translocate carrier vesicles enriched in these molecules from the perinuclear cytoplasm to sites along the myelin internode. Both (+)-end- and (−)-end-directed MT motors could potentially mediate transport from the perinuclear region (Kidd et al., 1993), but since most MTs have their (+) ends directed away from the perinuclear cytoplasm, it seems likely that most vesicles are delivered to myelin by (+)-end

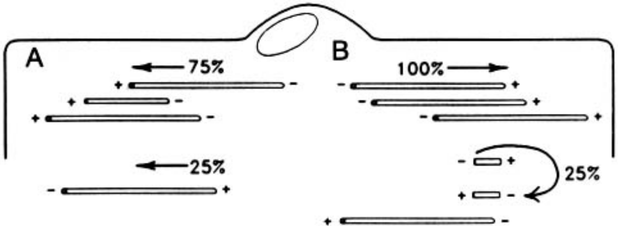

FIG. 42-8. Two possible mechanisms may generate microtubules of mixed polarity in the external cytoplasmic channels. (A) Microtubules may be transported in both orientations from the perinuclear region, although not in equal numbers. (B) Alternatively, microtubules may be initially transported from the perinuclear region with a uniform polarity, then depolymerize. About 25% become sufficiently short to rotate, and these subsequently elongate with their (+) ends toward the perinuclear region.

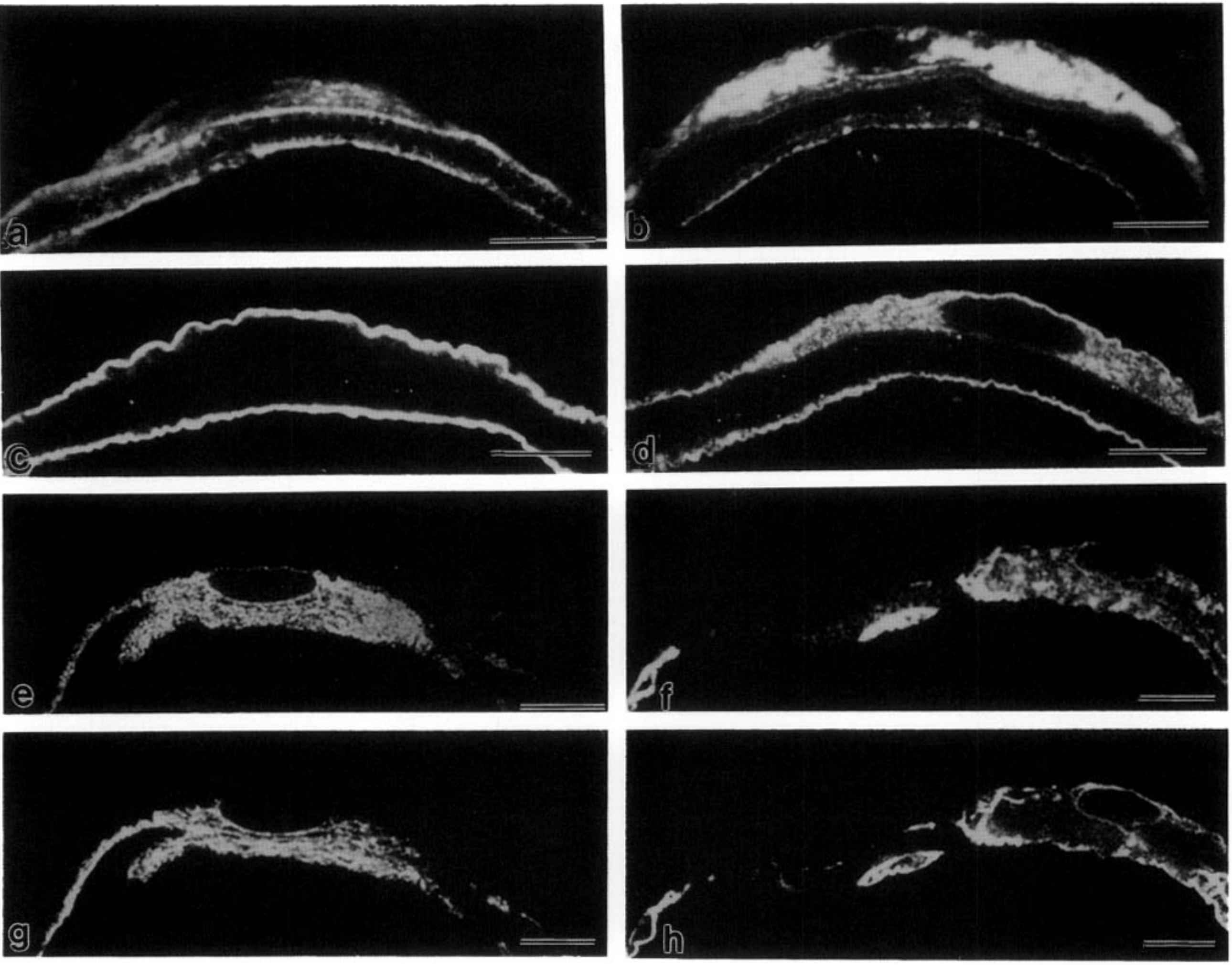

FIG. 42-9. Confocal fluorescence micrographs of myelinated nerve fibers from rat sciatic nerve, illustrating the effects of colchicine treatment on P_0 (*a, b*), laminin (*c, d*), endoplasmic reticulum (*e,f*), and vimentin (*g,h*) distributions (left panels, saline treated, right panels, 24-hour colchicine treatment). (*e,g*) and (*f,h*) show double-labeling of the same fibers. Note the islands of smooth endoplasmic reticulum that are surrounded by vimentin immunostaining after colchicine treatment (*arrows*). Scale bars = 10 μm. [From Trapp et al. (1993), with permission.]

MT motors. If so, what could be the function of MTs that have their (−) ends pointed toward the periphery? Translocation of mRNA and ribosomes has been attributed to (−)-end-directed transport in dendrites (Baas et al., 1988; Baas and Black, 1990), oocytes (Stebbings and Hunt, 1983; Yisraeli et al., 1990), and myelinating cells (Kidd et al., 1993). Translocation of MBP mRNA along the myelin internode is the chief means of delivering MBP to the myelin sheaths (Colman et al., 1982; Trapp et al., 1987; Griffiths et al., 1989) and previous studies have demonstrated that ribosomal RNA is transported from the nucleus to points along the peripheral nervous system myelin internode at a rate of 0.1 to 0.3 mm per day (Gould and Mattingly, 1990). Whether MBP mRNA is transported with ribosomes is unknown. Estimates of mRNA transport in other cells (0.1 to 0.5 mm per day; Yisraeli et al., 1990) suggest that cotransport is possible. If MBP mRNAs are transported attached to ribosomes, they must be very stable, with half-lives of days, or else they would be degraded before reaching the paranodes.

Do Schwann cell MTs play a role in targeting myelin proteins to distinct membranes of the myelin internode? Ultrastructural immunocytochemical studies indicate that P_0 and MAG are segregated into separate carrier vesicles as they exit from the *trans*-Golgi network (Trapp et al., 1993). The fate of these P_0- and MAG-rich carrier vesicles after MT disassembly has provided clues to the mechanisms of their targeting. Following MT disassembly, P_0-rich carrier vesicles fused with each other to form compact myelin-

like membrane whorls, while MAG carrier vesicles fused with each other to form membrane whorls distinct from those rich in P_0 protein. P_0 and MAG were not mistargeted to Schwann cell surface membranes after MT disassembly, suggesting that MTs do not play an essential role in directing these molecules specifically to compact myelin or the outer mesaxon. These data imply that each class of carrier vesicles contains signals that only permit their fusion with the correct surface membrane.

Organelle Distribution. The effects of MT disassembly demonstrate that MTs organize the distribution of Golgi membranes, endoplasmic reticulum, and intermediate filaments in myelinating Schwann cells (Hansson and Sjostrand, 1971; Jacobs et al., 1972; Roytta et al., 1984; Trapp et al., 1993) (Figure 42-9). Disruption of Golgi membranes after MT disassembly is consistent with similar studies in other cell types (Kreis, 1990; Karecla and Kreis, 1992) and indicates that MTs organize the Golgi apparatus by transport toward their (−) end. After MT disassembly, organelles aggregated at discrete sites within the cytoplasmic channels at the outer perimeter of the myelin internode. The organelle-rich regions consisted of a central core of smooth endoplasmic reticulum membrane surrounded by intermediate filaments. Other organelles were embedded in the intermediate filament-rich zone and excluded from the smooth endoplasmic reticulum-rich zone. These results indicate that MTs help organize independent networks of endoplasmic reticulum and intermediate filaments that extend from the perinuclear cytoplasm to the paranodal cytoplasm. Previous studies have demonstrated that certain lipids are synthesized in the smooth endoplasmic reticulum located in the outer cytoplasmic channels of the myelin internode (Gould and Sinatra, 1981; Gould et al., 1987). The interdependence between MTs and smooth endoplasmic reticulum extension (Trapp et al., 1993) may help coordinate the addition of lipids and proteins into compact myelin.

Axonal Modulation of Microtubule Organization in Myelinating and Degenerating Internodes

Schwann cells can display several phenotypes that are governed by axonal influences (Aguayo et al., 1976; Weinberg and Spencer, 1976). Physical contact with an appropriate axon induces expression of myelin protein genes (Lemke, 1986), whereas loss of axonal contact causes a rapid decrease in transcription of these genes (Trapp et al., 1988). Cell-cell contact influences the organization of cytoskeletal components in a variety of cell types (e.g., Bacallao et al., 1989), including Schwann cells (Kidd et al., 1993) (Figure 42-10).

In explant cultures of neonatal dorsal root ganglia, Schwann cells that have not yet myelinated and lack axonal contact radiate MTs from a single MT-organizing center that contains the centrosome (Kidd et al., 1993) (Figure 42-10). Thus, most MTs

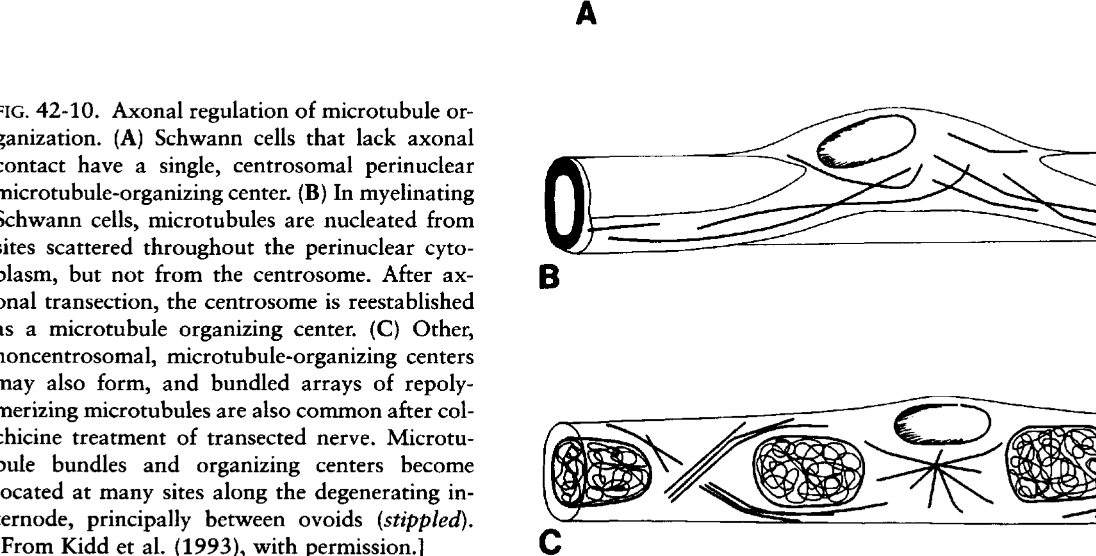

FIG. 42-10. Axonal regulation of microtubule organization. (A) Schwann cells that lack axonal contact have a single, centrosomal perinuclear microtubule-organizing center. (B) In myelinating Schwann cells, microtubules are nucleated from sites scattered throughout the perinuclear cytoplasm, but not from the centrosome. After axonal transection, the centrosome is reestablished as a microtubule organizing center. (C) Other, noncentrosomal, microtubule-organizing centers may also form, and bundled arrays of repolymerizing microtubules are also common after colchicine treatment of transected nerve. Microtubule bundles and organizing centers become located at many sites along the degenerating internode, principally between ovoids (*stippled*). [From Kidd et al. (1993), with permission.]

have their (+) ends oriented toward the cell periphery and their (−) ends at the center of the cell. This MT arrangement provides the Schwann cell with a simple transport system in which the Golgi apparatus and other (−)-end-tethered organelles are concentrated at the cell center, and transport to the cell surface is mediated by (+)-end-directed motors. MT-organizing centers were not observed in Schwann cells cultured from adult nerve or Schwann cells passaged many times, underscoring the dynamic nature of the Schwann cell MT network in different environments.

Contact with a myelin-inducing axon results in a more complex MT organization (Kidd et al., 1993) (Figures 42-6 and 42-10). As described above, the MT network in myelinating Schwann cells is formed from nucleating sites scattered through the perinuclear cytoplasm, and not from centrosome. A network of MTs with mixed polarity is generated in the external cytoplasmic channels, and most MTs are enriched in acetylated α-tubulin epitopes, a characteristic of stable MTs.

The dynamic nature of Schwann cell MTs and the influence of axonal contact on MT organization are further demonstrated by the pattern of MT organization during Wallerian degeneration (Kidd et al., 1993) (Figure 42-10). By 24 hours after axonal transection, Schwann cells in the distal stump form multiple MT-organizing centers and MT bundles. One perinuclear MT-organizing center contains the centrosome, while other MT-organizing centers and bundles of parallel MTs appear along the internode and are located between degenerating myelin ovoids. The MT-organizing centers and MT bundles are initially observed in perinuclear cytoplasm and later identified at sites along the internode, raising the possibility that axonal transection induces the transcription and peripheral transport of MT-nucleating materials. This MT organization occurs transiently and only occurs for 1 to 3 days after axonal transection. By 4 days after transection, Schwann cells disassemble their cytoplasmic MTs as they undergo mitosis. The observation that new MT organizing centers are produced in degenerating internodes and positioned between myelin ovoids (Kidd et al., 1993) provides evidence that Schwann cells participate in the early events of myelin degeneration. This MT distribution concentrates lysosomes and endosomes in periovoidal cytoplasm (Kidd and Trapp, unpublished observations), through their association with MT (−) ends (Matteoni and Kreis, 1987). These organelles may contribute hydrolytic enzymes to the degenerating myelin ovoids, and are likely to be involved in processing the products of myelin degeneration.

INTERMEDIATE FILAMENTS

IFs are polymers of one or more IF proteins and have a diameter of ~10 nm in electron micrographs. The IF proteins form six groups of structurally related proteins (for reviews, see Klymkowsky et al., 1989; Skalli and Goldman, 1991). In myelinating Schwann cells, the predominant IF protein is vimentin (Dahl et al., 1982; Schachner et al., 1984; Kobayashi and Suzuki, 1990). Several authors have reported glial fibrillary acidic protein in nonmyelinating (Remak) Schwann cells (Dahl et al., 1982; Jessen et al., 1984; Schachner et al., 1984; Fields and Yen, 1985; Kobayashi and Suzuki, 1990) indicating that axons mediate IF composition in Schwann cells. Neurofilament mRNAs and proteins have also been reported in Schwann cells (Kelly et al., 1992; Roberson et al., 1992). Although neurofilament proteins may coassemble with vimentin (Gill et al., 1990), they are not a major constituent of the cytoskeleton in normal myelinating Schwann cells. Recent studies in other cells have implicated phosphorylation of vimentin in the regulation of IF assembly and disassembly (Chou et al., 1989; Chou et al., 1990) and a vimentin-specific kinase has been identified (Chou et al., 1990). Little is known about the kinetics of IF assembly or disassembly in myelinating Schwann cells.

Intermediate filaments constitute a substantial component of the Schwann cell cytoskeleton (Kobayashi and Suzuki, 1990; Peters et al., 1991), but their organization and functions remain poorly understood. In myelinating Schwann cell, electron microscopy and immunocytochemical studies have identified IFs in the perinuclear cytoplasm and external cytoplasmic channels (Kobayashi and Suzuki, 1990; Peters et al., 1991; Trapp et al., 1993) (see Figure 42-9g). In the external cytoplasmic channels, IFs are oriented parallel to the axon and follow a sinuous course similar to MTs. While IFs in nonmyelinating Schwann cells appear bundled, those in myelinating cells are more widely spaced. This difference has been attributed to the presence of glial fibrillary acidic protein in nonmyelinating cells (Kobayashi and Suzuki, 1990). Intermediate filaments are generally considered to have a structural role in mechanically maintaining cell shape against externally applied forces (Klymkowsky et al., 1989; Skalli and Goldman, 1991). Vimentin IFs may interact with MF-associated molecules such as integrins (Wang et al., 1993) and ankyrin (Georgatos and Marchesi, 1985), and with MTs in resisting stress (Wang et al., 1993).

Are IFs essential for myelination? Because myelinating Schwann cells contain abundant IFs, and IF

composition between myelinating and nonmyelinating Schwann cells may vary, it is likely that IFs are important. Oligodendrocytes, however, make numerous myelin sheaths but have few, if any, IFs, suggesting that the presence of IFs is not a fundamental requirement of myelination. Both of these views may be correct, if the primary role of IFs is to support the Schwann cell against external stresses. Oligodendrocytes would not need IFs, since they are sheltered from external forces by the skull and vertebrae and by IF-rich astrocytes. Peripheral nerves, on the other hand, are frequently subjected to compression during limb movement and external trauma. While some of these forces are mitigated by the epineurium, perineurium, and the endoneurial collagen fibrils, an intracellular apparatus for maintaining cell shape and resisting external stress may also be necessary.

CONCLUSIONS

This chapter has reviewed the general distribution of microfilaments, microtubules, and intermediate filaments in myelinating Schwann cells and some of the effects of MT disassembly on the distribution of organelles and transport of myelin proteins. Myelinating Schwann cells offer many advantages for elucidating the role of (1) MFs in membrane movement and stabilization, (2) MTs in intracellular transport and organelle distribution, and (3) IFs in resisting external stress. The major challenge of the immediate future is to identify and characterize the molecules that are critical to cytoskeletal functions, such as MT motors or MF-binding proteins. Many of these molecules are likely to be expressed by other cell types and already characterized; immunocytochemical studies of Schwann cells with use of antibodies against known cytoskeletal components should be both productive and informative. Some cytoskeletal molecules, however, are likely to be Schwann cell-specific or at least specialized isoforms of known cytoskeletal protein families. The use of polymerase chain reaction technology may help identify and characterize molecules that have sequence homology to cytoskeletal molecules. The identification of unique molecules will require a dedicated search and a considerable amount of luck. Ultimately, successful application of transgenic technology and homologous recombination should elucidate the specific functions of the Schwann cell cytoskeleton and define the mechanisms responsible for spiral wrapping of myelin membranes and site-specific targeting of myelin proteins.

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43 | Intermediate filaments in astrocytes

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The cytoskeleton is composed of three major filamentous components; microfilaments (mainly actin), microtubules (mainly tubulins), and intermediate filaments (IFs). During the past ten years the chemistry, biology, and structure of the cytoskeleton and associated proteins have been described in numerous publications (Traub, 1985; Schliwa, 1986; Goldman and Steinert, 1990). Originally, five distinct classes of IF proteins were defined on the basis of the cell type or specific tissue from which the filaments were isolated and characterized: keratin in epithelial cells, vimentin in cells of mesenchymal origin, desmin in muscle cells, glial fibrillary acidic protein (GFAP) in astrocytes, and neurofilaments in neurons. Molecular biological studies based on amino acid homologies now show that all IF-forming proteins possess a central α-helix rod domain of closely conserved length and secondary structure, but with specific differences, which permit classification into distinct sequence types. At present, the IF proteins are divided into five types (Goldman and Steinert, 1990):

Type I: acidic keratins
Type II: neutral-basic keratins
Type III: includes vimentin, desmin, GFAP, and/or 57 kD neuronal IF protein
Type IV: classical neurofilaments
Type V: nuclear lamins

Vimentin, desmin, and GFAP are highly homologous throughout their rod and C-terminal domains.

This chapter focuses on the two principal IF proteins in astrocytes: vimentin and GFAP. The expression of vimentin and GFAP in developing and mature, differentiated astrocytes and following injury will be discussed. Since GFAP is the major IF protein in mature cerebral astrocytes, we have described *in vitro* and *in vivo* studies relating to GFAP metabolism, phosphorylation, assembly, immunocytochemistry, and molecular biology. The role of GFAP in gliosis and in brain tumor diagnosis has also been included. The chapter is intended to provide an overall summary of the various aspects of GFAP biology as it relates to astrocytes. Since an enormous amount of work has been done in this area, it has been impossible to include every relevant reference.

VIMENTIN

Vimentin was first demonstrated in chicken fibroblasts as the major subunit of filaments and is mainly expressed in cells of mesenchymal origin (Brown et al., 1976; Franke et al., 1979). Immunohistology showed that vimentin was present in a variety of cultured cells and permanent cell lines from other tissues. Cells of different embryologic origin produce vimentin when grown *in vitro*. Vimentin is also expressed sequentially during the differentiation of various cell lineages. Most cells *in situ* express only one type of IF. Exceptions include some glial cells, aortic smooth muscle cells, and neurons. For example, vimentin coexists with GFAP in immature astrocyte, in Bergmann glia and tanycyte processes (Shaw et al., 1981) and in Müller fibers of degenerating retinae (Shaw and Weber, 1983). It is thought that expression of vimentin is correlated with a less differentiated state. By immunofluorescence and electron microscopy, vimentin IFs occur as single filaments or gently curving, loose bundles. The genomes of various vertebrate cell types are known to have a single copy of a gene for vimentin (Quax et al., 1983) but this gene appears to make at least two mRNA species (Zehner and Paterson, 1983).

Vimentin in Central Nervous System Development and Reactive Astrogliosis

Developmental expression of cytoskeletal IFs has been studied in a number of experimental models. In the developing chick embryo, vimentin is present in virtually all of the replicating neuroepithelial cells in the early neural tube. As the cells mature, neurofilaments replace vimentin in the postmitotic neuron, and GFAP replaces vimentin in the mature astrocyte (Schnitzer et al., 1981; Tapscott et al., 1981). GFAP increases and vimentin decreases in differentiating rodent glial cells (Schnitzer et al., 1981). Vimentin and GFAP have been reported to coexist in varying proportions in cultured astrocytes (Schnitzer et al., 1981; Chiu and Goldman, 1984; Eng et al.,

1986a; Goldman and Chiu, 1984a) and in Bergmann radial glial fibers of the adult cerebellum (Schnitzer et al., 1981; Bovolenta et al., 1984). IF heteropolymers consisting of alternating GFAP and vimentin monomers have been demonstrated by immunoelectron microscopy in human glial tumors (Sharp et al., 1982; Wang et al., 1984) and cultured mouse astrocytes (Abd-El Basset et al., 1992). Studies by Dahl and coworkers (1982) have shown an increase of vimentin in the rat optic nerve undergoing Wallerian degeneration. Whether this increase is due entirely to activated and fibrous astrocytes or to other vimentin-containing cells, such as activated endothelial cells, macrophages, and lymphocytes, remains to be determined.

Schiffer et al. (1986) have studied astrocytic reactions to injury under two experimental conditions in the rat central nervous system: brains injured by laser irradiation and brain tumors induced with the chemical carcinogen, ethylnitrosourea. After immunostaining with antibodies to GFAP and vimentin, GFAP antibodies labeled all the reactive astrocytes, whereas vimentin antibody staining was found in astrocytes at the periphery of the lesion. The authors suggested that vimentin was expressed only in the astrocytes undergoing proliferation. Using the experimental allergic encephalomyelitis (EAE) model in the Lewis rat, we were unable to demonstrate the colocalization of GFAP and vimentin in the activated astrocytes, except possibly for a few proliferating astrocytes adjacent to perivascular lesions (Eng et al., 1986b). Under these conditions nonglial cells such as endothelial cells, macrophages, and lymphocytes were heavily immunostained for vimentin in freeze-substituted EAE rat spinal cord (L. Eng, M. Gibbs, and M. Smith, unpublished data). Proliferating astrocytes at the site of a stab wound in the brain or spinal cord contusion also immunostain for GFAP and vimentin (Calvo et al., 1991; L. Eng, unpublished observations).

GLIAL FIBRILLARY ACIDIC PROTEIN

GFAP studies originated from our research on multiple sclerosis, a demyelinating disease of the central nervous system that is characterized by intense reactive astrogliosis and scar formation. The term glial fibrillary acidic (GFA) protein evolved from "plaque" protein (Eng et al., 1970), glial fibrillary protein (Eng et al., 1971) to GFAP or GFA protein (Uyeda et al., 1972; Bignami et al., 1972). In the special issue of the *Journal of Neuroimmunology* (Vol. 8, No. 4–6) on GFAP Cedric Raine stated in his editorial that "this protein has become a prototype antigen in nervous tissue identification and a standard marker for fundamental and applied research at an interdisciplinary level" (Raine, 1985). Other protein preparations containing GFAP or its proteolytic degradation products are astroprotein (Mori and Morimoto, 1975) and alpha-albumin (Gheuens et al., 1984). A detailed discussion regarding GFAP-related proteins has been reported elsewhere (Eng, 1980). GFAP, first isolated from multiple sclerosis (MS) plaques (Eng et al., 1970, 1971), is the protein subunit of glial intermediate filaments, which form packed bundles within the cell bodies and cytoplasmic processes of differentiated astrocytes. In the past, considerable confusion existed regarding the chemical and immunologic relationships between the glial filament, neurofilament, and neurotubule proteins. Neurotubules had been reported to have similar chemical properties to glial filaments and GFAP (Johnson and Sinex, 1974; Dahl, 1976a, 1976b: Dahl and Bignami, 1976b; Chan et al., 1977). Neurotubules had also been reported to share common properties with neurofilaments (Wisniewski et al., 1968, 1971; Gaskin and Shelanski, 1976; Dahl and Bignami, 1977; Iqbal et al., 1977). Still other studies had suggested that neurofilament proteins had similar chemical and immunologic properties with GFAP and glial filaments (Davison, 1975; Dahl and Bignami, 1976a; Yen et al., 1976; Davison and Hong, 1977; Day, 1977; Lee et al., 1977; Goldman et al., 1978). Our early reports (De Vries et al., 1976; Eng et al., 1976b) indicating that GFAP from astrocytes was not related to neurofilaments were challenged but subsequently were confirmed by many studies (Bignami and Dahl, 1977; Liem et al., 1978; Schachner et al., 1978; Dahl and Bignami, 1979; Schlaepfer et al., 1979; Chiu et al., 1980). We direct those requiring more comprehensive treatment of the subject to the following reviews and books: Eng and Bigbee, 1978; Bignami and Dahl, 1979; Eng, 1979, 1980, 1985; Eng and DeArmond, 1982, 1983; DeArmond and Eng, 1984; Traub, 1985; Dahl et al., 1986; Schliwa, 1986; Eng and Shiurba, 1988.

Biochemical properties of GFAP that have impeded its characterization are insolubility in aqueous solvents, tendency to aggregate or polymerize, and susceptibility to neutral proteases. Only a very small pool of aqueous soluble GFAP could be detected at any age in development of the rat brain (Malloch et al., 1987), in cultured astrocytes (Chiu and Goldman, 1984), and in rat spinal cord (Aquino et al., 1988). Early reports of a soluble GFAP fraction utilized human postmortem tissue and animal tissue where the time between death and homogenization of the tissue was not controlled. The low molecular

weight soluble forms in these cases are probably due to a calcium-activated neutral proteinase, which has a high substrate specificity for vimentin and desmin (Schlaepfer and Zimmerman, 1981; Bigbee et al., 1983b; DeArmond et al., 1983a; Nelson and Traub, 1983; Ciesielski-Treska et al., 1984). Routine biochemical procedures are now available for isolating homogeneous preparations of GFAP from spinal cord homogenates in sufficient quantities for chemical, immunological, and metabolic studies (Chiu et al., 1980; Liem, 1982; Eng and DeArmond, 1983; Fukuyama et al., 1991). GFAP shows species-specific amino acid sequence heterogeneity, and its molecular weight ranges from 48,000 to 51,000 kD (Eng, 1980; DeArmond et al., 1983a), that is, 48,000 kD for mouse, 49,000 kD for human, 50,000 kD for bovine, and 51,000 for rat.

Metabolism

Metabolic studies with rodent astrocyte cultures have shown that the rate of GFAP monomer synthesis and cytoplasmic accumulation of glial filaments can be experimentally modulated (Chiu and Goldman, 1985). Goldman and Chiu (1984a) were able to alter the morphology of neonatal rat cortical astrocytes by varying the initial seeding densities. Astrocytes in low-density cultures were flat and polygonal in shape, and they contained large amounts of actin relative to IF proteins. In contrast, astrocytes in high-density cultures appeared morphologically differentiated, often were stellate in shape, and had delicate, branching cytoplasmic processes. Astrocytes at high density contained relatively less actin but more IF proteins than astrocytes in low-density cultures. Chiu and Goldman (1984) did not detect any influence of growth rate on the pattern of IF protein synthesis, and they found that the turnover of GFAP revealed both a fast-decaying pool (half-life: 18 hours) and a more stable form (half-life: 8 days). These authors (Goldman and Chiu, 1984b) reported that the intracellular content of GFAP could be doubled by exposing cultured astrocytes to dibutyryl-cyclic AMP (cAMP) for 2 weeks. The increase in GFAP content also was correlated with a reduction in cytoskeletal actin and morphological transformation of the flat, polygonal astrocyte to the stellate form. cAMP (Sensenbrenner et al., 1980), beta-adrenergic agonists such as isoproterenol (McCarthy et al., 1985; Pollenz and McCarthy, 1986), and agents such as phorbol esters that activate protein kinase C (PKC) (Mobley et al., 1986; Mobley and Harrison, 1991) also have been found to transform the flat, polygonal astrocyte to the stellate form. Morrison et al. (1985) observed that growth factors and hormones regulate the expression of GFAP *in vitro*. Induction of GFAP synthesis in secondary rat astrocyte cultures grown in chemically defined medium increased GFAP content 2- to 4-fold in the presence of insulin, hydrocortisone, putrescine, prostaglandin $F_{2\alpha}$, and pituitary fibroblast growth factor. Increase in the specific rate of GFAP synthesis was maintained for up to 3 weeks after the cells were exposed to chemically defined medium. The resultant elevation in GFAP content accompanied the morphological maturation of astrocytes from the flat, polygonal to the stellate form. The half-life for GFAP was determined by two independent methods to be 7.5 days. Conversion of the cultured cells to chemically defined medium did not result in an increase in intracellular levels of cAMP (Wu et al., 1985). Astrocytes cultured in serum-containing medium grow to confluence, stop producing GFAP, and remain morphologically undifferentiated as flat cells with few processes. If these cells are grown in serum, but on a nitrocellulose membrane, at confluence they continue to differentiate into fibrous process-bearing astrocytes, which continue to increase in GFAP content. This suggests that, in addition to growth and matrix factors, there is a physical component that can induce astrocyte differentiation (Eng et al., 1986a).

In contrast, GFAP turnover *in vivo* in mouse spinal cord (DeArmond et al., 1986) was much slower than that reported for astrocytes in culture (Chiu and Goldman, 1984; Morrison et al., 1985). At 9 weeks after intravenous injection of [guanido ^{14}C]arginine, 40% of the radioactivity incorporated into spinal cord cytoskeletal GFAP was still present, indicating that a significant proportion of glial filaments is degraded relatively slowly *in vivo* (DeArmond et al., 1983b, 1986). In addition, study of short-term *in vitro* uptake of [^{3}H] amino acids into rat spinal cord IF proteins showed that GFAP had a slower rate of turnover than any of the neurofilament proteins (Smith et al., 1984b). The relatively slow turnover rate for GFAP is consistent with a possible structural role for glial filaments.

Phosphorylation

Posttranslational phosphorylation may account for the charge heterogeneity of GFAP observed following isoelectric focusing by polyacrylamide gel electrophoresis. *In vitro* translation of GFAP mRNAs from normal and mutant rodent central nervous system and from a human glioma-derived cell line grown in culture and as a solid tumor yielded single

molecular weight polypeptides, which showed ionic charge differences among two to three spots with an isoelectric pH range of 5.7 to 5.9 (Bigbee and Eng, 1982). Norephinephrine treatment of C6 glioma cells induced the phosphorylation of a number of intracellular proteins, including vimentin and GFAP (Browning and Ruina, 1984). All receptor agonists that have been shown to increase cAMP levels increase phosphorylation of GFAP and vimentin in cultured astrocytes (McCarthy et al., 1985). While cAMP treatment of astrocytes induces a morphological change from the flat to process-bearing cell (Sensenbrenner et al., 1980), phosphorylation of GFAP and vimentin is independent of the morphological change (Pollenz and McCarthy, 1986).

Phorbol ester-induced changes in astrocyte morphology and GFAP and vimentin phosphorylation produce a shift in PKC from the cytosol to the membrane (Harrison and Mobley, 1990). Both the PKC activator, phorbol 12-myristate 13-acetate (PMA), and the cAMP-dependent protein kinase (PKA) activator, 8 bromo-cyclic AMP, phosphorylates several regions of GFAP and vimentin (Harrison and Mobley, 1991). ATP-evoked calcium signal stimulates protein phosphorylation/dephosphorylation in cultured astrocytes. The phosphorylation of a 52 kD protein, which comigrates with GFAP in sodium dodecyl sulfate polyacrylamide gel electrophoresis, supports the premise that calcium-dependent protein kinases and phosphatases are transducing elements for calcium signal brought about by activation of P2 purinergic receptors in astrocytes (Neary et al., 1991). PMA and inhibitors of PKC have been shown to alter GFAP mRNA (Sharma et al., 1991). A cytoskeletal-associated protein activity has been identified that phosphorylates GFAP and vimentin and is distinct from PKC and PKA (Harrison and Mobley, 1992).

Monoclonal antibodies to two synthetic peptides were produced, which react to phosphorylated GFAP. pG1 reacted to the serine in residues 3-13 and pG2 reacted to residues 29-39. The phosphorylation of these two serine residues on intact GFAP induced disassembly of glial filaments *in vitro* (Inagaki et al., 1990). These two antibodies react specifically with mitotic astroglial cells. The authors suggest that increased phosphorylation during mitosis may directly influence intracellular organization of glial filaments (Nishizawa et al., 1991; Matsuoka et al., 1992).

GFAP was found to be phosphorylated *in vivo* after intracerebral injection of ^{32}P-orthophosphate, in brain slices and in a cell-free system. In both systems, the GFAP was phosphorylated at the serine and threonine residues. Incubation of brain slices with ^{32}P and the PKC activator, PMA, or an activator of cAMP-dependent protein kinase, forskolin, stimulated phosphorylation of GFAP. Phosphorylation of GFAP was also enhanced by calcium/phosphatidylserine/diolein and by exogenous cAMP-dependent kinase in a cell-free system. These findings indicate that PKC and cAMP-dependent kinase may play physiological roles in the *in situ* phosphorylation of GFAP. In cytoskeletal preparations incubated with [γ^{32}P]ATP, GFAP was phosphorylated *in vitro* by two additional protein kinases, a Ca^{2+}/calmodulin-dependent and an effector-independent kinase. These data suggest that phosphorylation of GFAP may be regulated by multiple second-message pathways (Noetzel, 1990a). A brain slice preparation was also employed to study GFAP synthesis and phosphorylation. The results indicate that the increase of GFAP during the first month of life cannot be ascribed solely to the rate of GFAP synthesis. The findings are consistent with the hypothesis that during later stages of astrocytic development the accumulation of GFAP may be due to a low rate of protein degradation. The pattern of GFAP phosphorylation in the developing rat brain differed from that observed for the incorporation of [^{3}H]amino acids into GFAP. The peak incorporation of ^{32}P into GFAP occurred on postnatal day 10 at a time when synthesis of the protein had declined by 43%. These results suggest that during GFAP development, phosphorylation of GFAP is mediated by factors different from those directing its synthesis (Noetzel, 1990b).

Assembly

A number of *in vitro* assembly studies of GFAP have reported that cations, pH, ionic strength, and ATP influence assembly (Rueger et al., 1979; Lucas et al., 1980a, 1980b; Yang et al., 1988; Yang and Babitch, 1988; Quinlan et al., 1989; Steward et al., 1989). A recent report employing a fluorescence energy transfer assay method suggests that GFAP filaments appear to be in dynamic equilibrium with subunits *in vitro* (Nakamura et al., 1991). These authors conclude that factors other than pH, ionic strength, or metal ions modulate the assembly state of GFAP *in vivo*, one of the factors being phosphorylation. As mentioned previously, all IF proteins share a common subunit organization consisting of a non-α-helical N-terminal head domain, a central α-helical rod domain, and a C-terminal tail domain (Weber and Geisler, 1985). The *in vitro* assembly process of IFs seems to consist of several association steps that involve different protein domains. Dimer and tetramer formation depend solely on the α-helical rod domain (Kaufmann et al., 1985). Assembly of filaments from

tetramers requires the presence of the head domain (Traub and Vorgias, 1983; Kaufmann et al., 1985). The head domain of the different IFs are variable in size and amino acid sequence; however, they are positively charged, because of a series of arginine residues, and contain serine residues available for phosphorylation by PKA, PKC, and cdc2 kinase (Inagaki et al., 1988). *In vitro* studies suggest that phosphorylation of vimentin (Inagaki et al., 1987; Evans, 1988), desmin (Geisler and Weber, 1988; Inagaki et al., 1988), and GFAP (Inagaki et al., 1990) inhibit IF assembly. It has been proposed that phosphorylation could participate in the regulatory processes in assembly and turnover of IFs (Geisler and Weber, 1988).

Molecular Biology

A clone encoding mouse GFAP was initially reported by Lewis et al. (1984). The mouse GFAP gene was first located on chromosome 11 by Bernier et al. (1988) and more recently confirmed by Boyer et al. (1991) and Brownell et al. (1991). The chromosomal location of the human GFAP gene was located on chromosome 17q21 by Bongcam-Rudloff et al. (1991) and also confirmed by Brownell et al. (1991) and Kumanishi et al. (1992). cDNA clones encoding rat GFAP were isolated from rat astrocyte and Schwann cell cultures. Nucleotide sequences from astrocytes and Schwann cells contained identical coding, however the 5′ untranslated region from the Schwann cell line indicated that the start site for peripheral nervous system GFAP mRNA lies 169 bases upstream from that used in the central nervous system. The data suggest that structural differences between GFAP in these two cell types occur at the nucleic acid and protein level (Feinstein et al., 1992).

The studies of promoter and enhancer elements of GFAP gene provide an understanding of GFAP regulation in glial development and glial response to injury. cDNA clones encoding human GFAP were isolated which contain the complete GFAP coding region (Reeves et al., 1989; Brenner et al., 1990). *In vitro* transcription analysis showed that the basal level expression of GFAP is controlled by two elements: a TATA box located about 25 base pairs (bp) upstream from the transcription start site, and another element located between +11 and +50 bp downstream from the start site (Nakatani et al., 1990a, 1990b). Miura et al. (1990) found that the *cis* element for astrocyte-specific expression was located within 256 bp from the transcription startpoint. They further defined three *trans*-acting factor binding sites: AP-2, NFI, and cAMP-responsive element motifs, which explains upregulation of GFAP in response to various kinds of injury. Transient transfection studies with a chloramphenicol acetyl transferase reporter gene were used to identify three regions (A, B, and D) responsible for GFAP gene expression (Besnard et al., 1991). The D region is located near the basal promoter, which is similar to the finding of Miura et al. (1990), while A and B are next to each other about 1500 bp further upstream. Employing site-directed mutgenesis of the 124-bp B region, the same group from Brenner's laboratory showed that there are multiple active sites in this region. Activation of GFAP transcription in astrocytes involves interaction among factors binding to these sites. The most crucial sequence has been shown to be a consensus AP-1 binding site. This is the first demonstration for a function of a specific transcription site in astrocytes, since polyclonal antibodies to c-Fos and c-Jun recognize proteins binding this site (Masood et al., 1993). Sarkar and Cowan (1991) found two similar positive elements, one is located −1.631 to −1.479 bp and another one located −97 to −80 bp; however, they also found a negative element that is located within the first intron of the GFAP gene. More recently, Kaneko and Sueoka (1993) found two negative regulatory regions: GDR1 is in a 2.7-kb region extending from the first intron through the fifth exon, and GDR2 is within 1.7 kb 3′ of the polyadenylation site. GDR1 alone inhibit GFAP expression in nonneuronal tissues, while both GDR1 and GDR2 inhibit the GFAP expression in neuronal cells.

GFAP In Development

In situ hybridization with a GFAP cDNA probe applied to the developing mouse brain has shown that mRNA levels with the astrocytes vary by a large amount depending on their location. In the adult brain, the astrocytes in the glial limitans contain the highest amount of GFAP mRNA with a decreasing amount localized to the white matter and gray matter (Lewis and Cowan, 1985). Employing Northern blot analysis with a GFAP cDNA probe (Lewis and Cowan, 1985), Landry et al. (1990) found that GFAP mRNA expression followed a caudal to rostral gradient, consistent with overall brain development. In a recent transcriptional study with a cDNA probe to GFAP, during mouse brain maturation, GFAP-mRNA occurred in a two-step developmental expression. It increased between birth and day 15 and then decreased until day 55 (Tardy et al., 1989; Riol et al., 1992). In a combined immunocytochemical and *in situ* hybridization study of the GFAP and

GFAP mRNA in the developing mouse retina, it was found that Müller cells do not express or contain low amounts of GFAP. GFAP-containing cells were found only in the ganglion cell and nerve fiber layers after postnatal day 1 and continue to form until P-10. GFAP-mRNA levels were high in the first week of birth and declined rapidly as the animal developed. During normal retinal development, GFAP and GFAP-mRNA were present only in astrocytes (Sarthy et al., 1991). A recent study by Erickson et al. (1992) reported that by RNA blotting analysis, normal cat retina expressed a low basal level of GFAP mRNA, which was induced 500% within 3 days of retinal detachment. Electron microscopic *in situ* hybridization analysis detected GFAP mRNA in detached retinas, but not in normal retinas. GFAP mRNA was readily detected in retinal astrocytes.

Developmental expression of GFAP mRNA has been reported in a number of astrocyte culture studies. During astroglial proliferation in culture (7 to 18 days), GFAP mRNA increased. A decrease followed with marked changes in cell shape, cell process outgrowth, and the accumulation of glial filaments (Charriere-Bertrand et al., 1989; Tardy et al., 1989); cAMP-stimulating agents increase GFAP levels (Hertz et al., 1978; Sensenbrenner et al., 1980; Goldman and Chiu, 1984b), while short-term treatment with cAMP-induced cell shape changes and no increase in GFAP. Increase in GFAP mRNA occurred only with long-term treatment (Le Prince et al., 1991). cAMP-dependent kinase and PKC may be regulating roles in determining GFAP mRNA levels (Shafit-Zagardo et al., 1988; Messens and Slegers, 1992).

GFAP In Reactive Gliosis

A timely, excellent review on the molecular profile of the reactive astrocyte documents the broad and continuing interest in astrogliosis. The numerous *in vitro* and *in vivo* studies of molecules, which are upregulated during astrocyte activation, illustrates their complex and varied responses (Eddleston and Mucke, 1993). Reactive astrogliosis is a prominent feature of astrocytes adjacent to and extending far beyond the site of injury. It occurs in central nervous system demyelination such as in MS and in degenerative diseases such as Alzheimer's disease, Creutzfeldt-Jakob's disease, and Huntington's disease. Gliosis is characterized by astrocyte proliferation and extensive hypertrophy of the cell body and cytoplasmic processes. Activated, reactive astrocytes exhibit cytological, biochemical, and histochemical transformations which include increases in nuclear diameter, elevated DNA levels, heightened oxidoreductive enzyme activity, and increased synthesis of GFAP, vimentin, glutamine synthetase, and glycogen (Eng and Shiurba, 1988). However, the biochemical events that precede and trigger astrocyte activation are unknown, and the proposed inhibitory effects of glial scarring on central nervous system repair, including neuronal regeneration and remyelination, remain unexplained at the molecular level.

Astrogliosis is thought to play a role in the healing phase following central nervous system injury by actively monitoring and controlling the molecular and ionic contents of the extracellular space of the central nervous system. Important extracellular constituents that may be regulated by reactive astrocytes include potassium ions, neurotransmitters, trophic factors, nutrients, and metabolic waste products. (For reviews, see Kimelberg and Ransom, 1986; Manthrope et al., 1986; Reier, 1986). Astrocytes participate in the removal of myelin and neuronal debris from injured areas. They also encapsulate regions of the central nervous system that are exposed to non-central nervous system tissue environments following trauma. In contrast to a role in healing central nervous system injury, gliosis may produce pathological effects by interfering with the function of residual neuronal circuits, by preventing remyelination, or by inhibiting axonal regeneration (Reier et al., 1983; Reier, 1986). It is therefore important to identify the essential molecular mechanisms that activate alternative metabolic responses in astrocytes in order to understand the pathogenesis of neuropathological lesions. These signals may result from anoxia due to disruption of the blood supply; dilution of inhibitory "chalones" around the injury site due to edema; changes in the ionic and molecular composition of the central nervous system extracellular fluid after disruption of the blood-brain barrier; release of growth factors and cytokines from non-central nervous system cells such as macrophages, T and B cells that may infiltrate the lesion; and loss of ionic coupling and release from cell density-dependent inhibition of growth due to increase in extracellular space. The signals also include factors from activated endothelial cells and microglia at the site of the lesion and secretion of mitogenic and trophic factors from degenerating neurons and oligodendroglia.

Astrogliosis is characterized by rapid synthesis of GFAP intermediate filaments and by hypertrophy of the astrocytic cytoplasmic processes. One can only speculate about the functional significance for this increase in IFs. Evidence from studies with rat optic nerve astrocyte cultures suggests that content and

subcellular distribution of IFs are important for cytoplasmic process formation and for structural stability of astrocytes. As mentioned previously, the relatively slow metabolic turnover rate for GFAP is consistent with such a structural role (DeArmond et al., 1983b, 1986; Smith et al., 1984b). Ultrastructural and immunocytochemical studies of astrocytic differentiation *in vitro* show that the flat, polygonal astroblast contains abundant microtubules and actin stress fibers; however, these elements progressively decrease, while GFAP increases during the change in shape of this astroblast to a stellate cell having slender, unbranched processes (Ciesielski-Treska et al., 1982a, 1982b; Trimmer et al., 1982).

Increased protein content or immunostaining of GFAP has been found in experimental models involving gliosis, such as the cryogenic lesion (Amaducci et al., 1981), stab wounds (Latov et al., 1979; Mathewson and Berry, 1985; Takamiya et al., 1986, 1988; Jeneczko, 1988; Miyake et al., 1988; Topp et al., 1989; Hozumi et al., 1990b; Vijayan et al., 1990), toxic lesions (Brock and O'Callaghan, 1987; Reinhard et al., 1988; Rataboul et al., 1989), and experimental allergic encephalomyelitis (EAE) (Smith et al., 1983, 1984a; Goldmuntz et al., 1986; Aquino et al., 1988).

Increase of GFAP-mRNA has been found in brains infected with scrapie and Alzheimer's disease (Wietgrefe et al., 1985); Creutzfeldt-Jakob disease (Manuelidis et al., 1987); in 6-hydroxydopamine lesion of the substantia nigra in the rat (Rataboul et al., 1988, 1989), in a mechanical lesion to rat cerebral cortex (Condorelli et al., 1990; Hozumi et al., 1990a; Landry et al., 1992); in entorhinal cortex lesions (Poirier et al., 1991); corticospinal axotomy (Kost-Mikucki and Olbinger, 1991), in EAE (Aquino et al., 1990), and in the lesioned dentate gyrus (Steward et al., 1990).

Transgenes

A transgenic vector containing almost the entire GFAP gene plus 5′ and 3′ flanking regions was fused to the *E. coli lacZ* structural gene. Injection of the GFAP-*lacZ* hybrid gene into the germline of mice yielded six different lines of transgenic mice. The expression of *lacZ* was astrocyte-specific. Upmodulation of transgene expression showed that induction of GFAP-*lacZ* expression was detectable within 1 hour after a focal mechanical lesion (Mucke et al., 1991). In a recent study, transgenic mice carrying the *lacZ* reporter gene linked to a 2.2 kb 5′ flanking sequence derived from the human GFAP gene were produced. This promoter directed expression to astrocytes and was also upregulated following injury to the brain. This approach can now be used to target expression of other heterologous genes to astrocytes *in vivo,* and to study the mechanism for reactive gliosis at the DNA level (Brenner et al., 1993). Cultured astrocytes prepared from GFAP *lacZ* transgenic pups implanted into brains of nontransgenic mice migrated to specific regions of the host brains and expressed the GFAP *lacZ* fusion gene for at least a year. Graft-derived astrocytes responded to focal injuries in these regions, but did not invade neural lesions in other areas. Their predictable migration and prolonged injury-responsive expression of GFAP driven transgenes should make these astrocytes potent vehicles for delivery of therapeutic agents (Mucke and Rockenstein, 1993).

Immunochemistry

GFAP and the other IFs share some chemical properties and common intramolecular polypeptide domains, however, GFAP also has some unique, highly immunogenic epitopes. We have produced specific GFAP antibodies in a rabbit immunized with MS plaque tissue that had been fixed in formalin for several years. These GFAP-specific epitopes are detected by high-affinity polyclonal antisera and monoclonal antibodies prepared from human and bovine GFAP. GFAP immunoreactivity in the mature central nervous system is restricted to glial filaments within protoplasmic astrocytes in gray matter, fibrous astrocytes in white matter, and radial glia in the cerebellum (Bergmann glia) and subependymal astrocytes adjacent to the cerebral ventricles. At the surface of the brain, GFAP immunoreactivity is especially concentrated in astrocytes which form the outer limiting membrane, the glia limitans. Polyclonal and monoclonal antibodies to GFAP have been extensively used for immunochemical and immunocytochemical studies (for reviews and discussions see Eng and Bigbee, 1978; Eng, 1979, 1980; Bignami and Dahl, 1979; Eng and DeArmond, 1983; Dahl and Bignami, 1983, 1985; Eng, 1985; Dahl et al., 1986; Eng et al., 1987.)

Mild tissue processing methods (i.e., unfixed, frozen, or freeze-substituted sections) and more sensitive detection procedures (immunogold labeling) have demonstrated GFAP-like immunoreactivity in regenerating teleost spinal cord, in Schwann cells, glialike cells in the myenteric plexus, Kupffer cells of the liver, salivary tumors, a pineal astrocytoma, and cells in the pineal gland. GFAP immunoreactivity has also been demonstrated in epiglottic cartilage, pituicytes and pituitary adenomas, immature oligo-

dendrocytes, papillary meningiomas, and metastasizing renal carcinomas. Mouse lens epithelium reacts with both polyclonal and monoclonal anti-GFAP antibodies.

Immunochemical application of GFAP antiserum has been used to quantitate GFAP in a variety of immunoassays. These include quantitative immunoelectrophoresis, competitive radioimmune assays, 2-site immunoradiometric assays, and enzyme-linked immunoassays (ELISA). Anti-GFAP antisera have been employed to isolate translated GFAP *in vitro* (Beguin et al., 1980), to immunostain and identify GFAP in transblots, and to identify reassembled GFAP fibers *in vitro* (Eng and Shiurba, 1988).

GFAP antibodies have been used extensively to identify astrocytes in normal, pathological, and experimental tissue. One must be cautious in interpreting immunocytochemical results. While positive staining may identify an astrocyte in the central nervous system, a negative result may be false. It is important to remember that this morphological technique is only indirect evidence for the presence of an antigen. The method of tissue processing is a very important factor. For example, the protoplasmic astrocyte in normal gray matter has a low content of GFAP and does not stain for GFAP when fixed in formaldehyde for an extended period of time. However, pretreatment of the formaldehyde-fixed tissue section with trypsin exposes more GFAP epitopes, which then can be detected by immunostaining. This has been confirmed on freeze-substituted brain tissue (see discussion in DeArmond and Eng, 1984). A much larger number of astrocytes immunostain for GFAP in the granule cell layer and white matter of the normal mouse cerebellum when the tissue is fixed by freeze-substitution instead of aldehyde (Figure 43-1). Monoclonal antibodies to GFAP have been generated that are specific and others that bind to both GFAP and vimentin (Eng, 1985; Pegram et al., 1985).

The intensity of GFAP immunostaining does not always correlate with GFAP content. Two examples are the rapid increase in GFAP staining seen 30 minutes after a cryogenic lesion of the rat brain (Amaducci et al., 1981) and that seen in the early stages of experimental allergic encephalomyelitis (EAE) (Smith et al., 1983). EAE is a cell-mediated autoimmune disease, which has been the principal experimental model for studying the etiology and pathogenesis of MS (Smith et al., 1984a; Eng et al., 1986b; Smith et al., 1987; Smith and Eng, 1988; Smith et al., 1988). Astrocyte proliferation and hypertrophy of cell processes appear very early in this model, coincidentally with the first inflammatory foci and is apparent by an increase in immunostaining of the astrocytes with GFAP antibodies without an increase in GFAP content (Smith et al., 1983; Goldmuntz et al., 1986). Aquino et al. (1988) have shown that this increase in GFAP immunoreactivity of the astrocytes without a corresponding increase in GFAP content is not due to an increase in GFAP epitopes resulting from limited proteolysis, to glial filament dissociation yielding an aqueous soluble fraction, or to differences in the avidity of a number of different antibody preparations tested. In acute EAE, the increase in GFAP immunostaining of the astrocytes is widespread and not confined to lesion sites (Smith et al., 1983). The reason for astrocyte hypertrophy and increased immunostaining for GFAP without a demonstrable increase in GFAP content is unknown. The onset of edema due to disruption of the blood-brain barrier and leakage of bloodborne substances into the central nervous system (Cutler et al., 1967; Juhler et al., 1984) may contribute to this phenomenon. Swollen astrocytic processes filled with disrupted bundles of glial filaments and glycogen particles have been shown by electron microscopy in edematous brain tissue (Kimelberg et al., 1982).

Recently we examined the early EAE lesion by electron microscopy and obtained results similar to that reported by Kimelberg et al. (1982). The astroglial processes contained many glycogen particles. The glial filaments were arranged in small bundles or loose thin filaments adjacent to the bundles. The glial filaments that normally appear as tight bundles expanded and appeared less dense (Figures 43-2 and 43-3). The general picture indicated that the first stages of EAE are pathogenetically related to an abnormal blood-brain barrier permeability. The "watery" cytoplasm of astrocytes at this early stage of EAE is most likely expressing a "partial" breakdown of the blood-brain barrier, resulting in intracellular (astrocytic) edematous fluid (Lee, 1982; Miquel et al., 1982). We have suggested that the increase in GFAP immunostaining, without an increase in GFAP content, is due primarily to the disruption of the blood-brain barrier. The resulting edema allows the tight bundles of glial filaments to dissociate and thus expose more antigenic sites (epitopes) to GFAP antibodies (Eng et al., 1989). GFAP intermediate filament dissociation can also explain the rapid increase in GFAP staining following cryogenic lesion of the rat brain without an increase in GFAP content (Amaducci et al., 1981).

Clinical Studies

Since the initial reports describing the use of GFAP antiserum for tumor diagnosis (Deck et al., 1976;

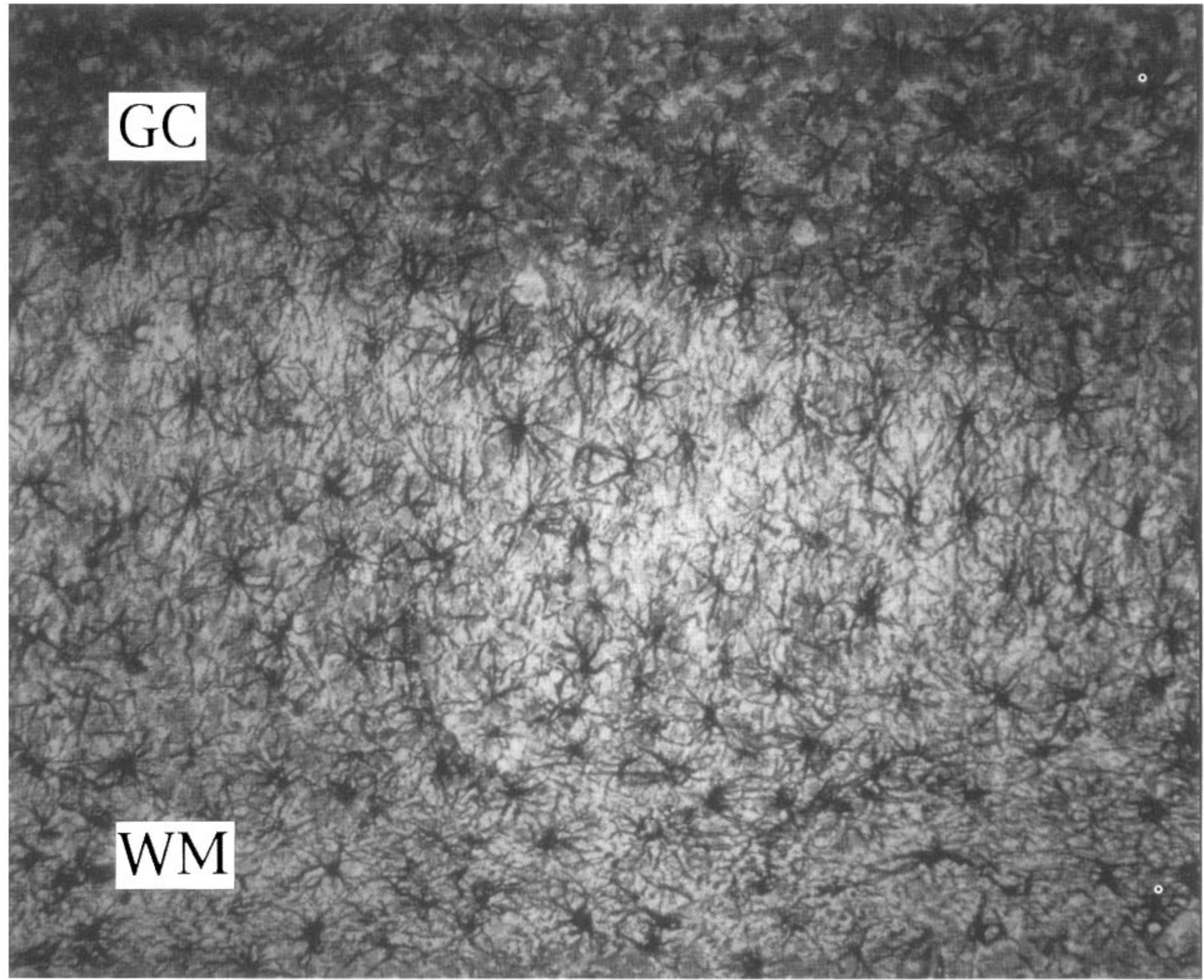

FIG. 43-1. A segment of mouse cerebellum was freeze-substituted in a mixture of chloroform, methanol, and acetone; the tissue was embedded in paraffin; a 5- to 6-μm section was immunostained with a polyclonal antiserum to human GFAP; and the GFAP visualized with the colloidal gold-silver enhancement technique. Note the dark silver-stained astrocytes in the granule cell layer (*GC*) and white matter (*WM*).

Duffy et al., 1977; Deck et al., 1978; Eng and Rubinstein, 1978), numerous reviews have appeared (DeArmond et al., 1980; Eng, 1980; DeArmond and Eng, 1984). Polyclonal and monoclonal antibodies to GFAP are used routinely in medical centers throughout the world to assist in the diagnosis of human neoplasms. It is always better to use a specific polyclonal antibody than a single monoclonal antibody for tumor diagnosis. A cocktail of three different monoclonal antibodies has been used successfully for identifying GFAP-containing tumor cells (McLendon et al., 1986).

Immunocytochemical assays, radioimmunoassays, and ELISA have been used to examine cerebrospinal fluid and body fluids from patients with MS, brain tumors, stroke, Alzheimer's disease, and other neurological diseases. GFAP has become an immunohistochemical marker for determining glial origin of many human brain tumors, and it may also provide a clinically useful tool for studying nonneoplastic central nervous system disorders.

FUTURE PROSPECTS

Morphological and metabolic studies indicate that GFAP may function by providing structural stability to the astrocyte and its processes. Other possible roles have not been demonstrated; however, many possible factors and biochemical mechanisms that may induce astrocyte activation are being studied. This reflects the availability of *in vitro* models, highly sensitive and specific analytical methods, monospecific antibodies, and cDNA probes. These

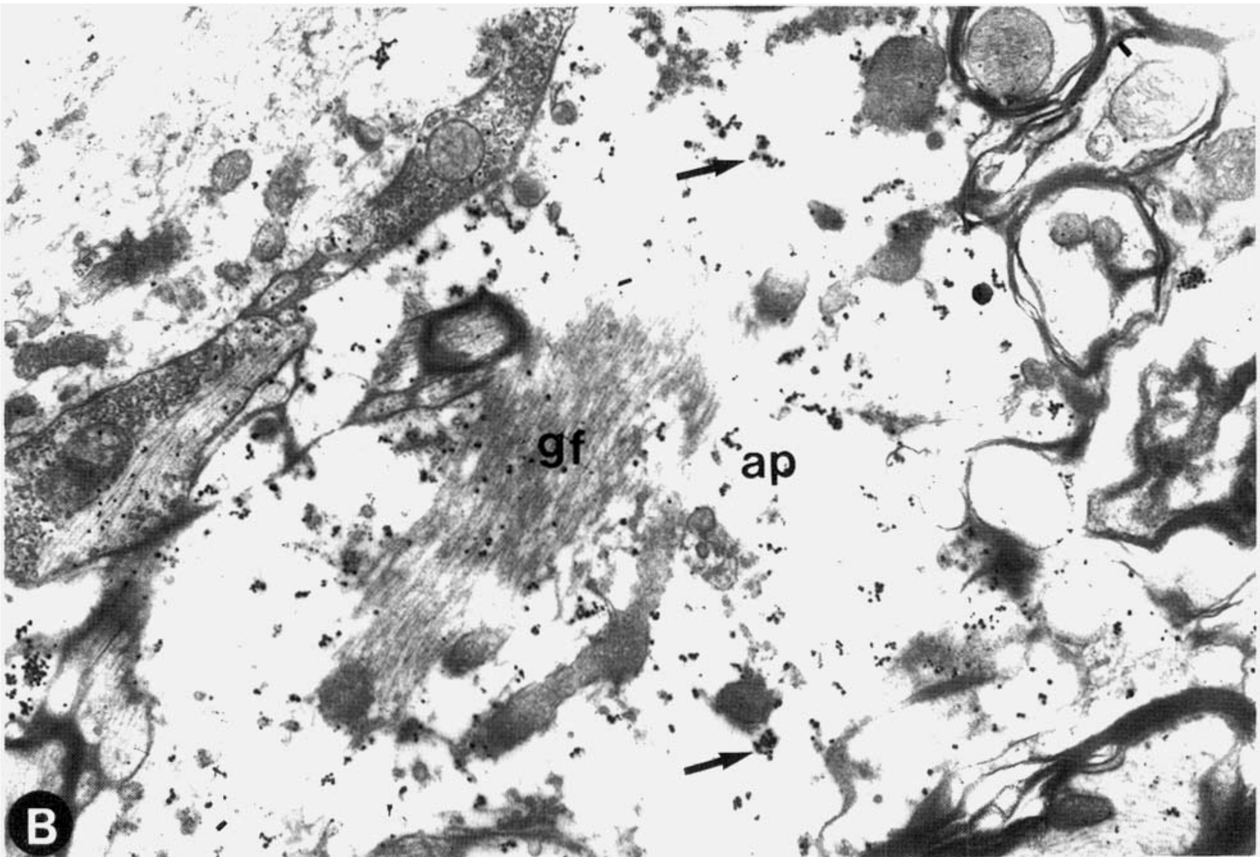

FIG. 43-2. Experimental allergic encephalomyelitis. Lumbar spinal cord. (**A, B**) Swollen astrocytic processes (*ap*) showing glycogen particles (*arrows*) and dispersed glial filaments in Figure A (*arrowheads*) and a small tight bundle (*gf*) in Figure B. Figure A: ×18,750; Figure B: ×15,390. [From Eng et al. (1989), with permission.]

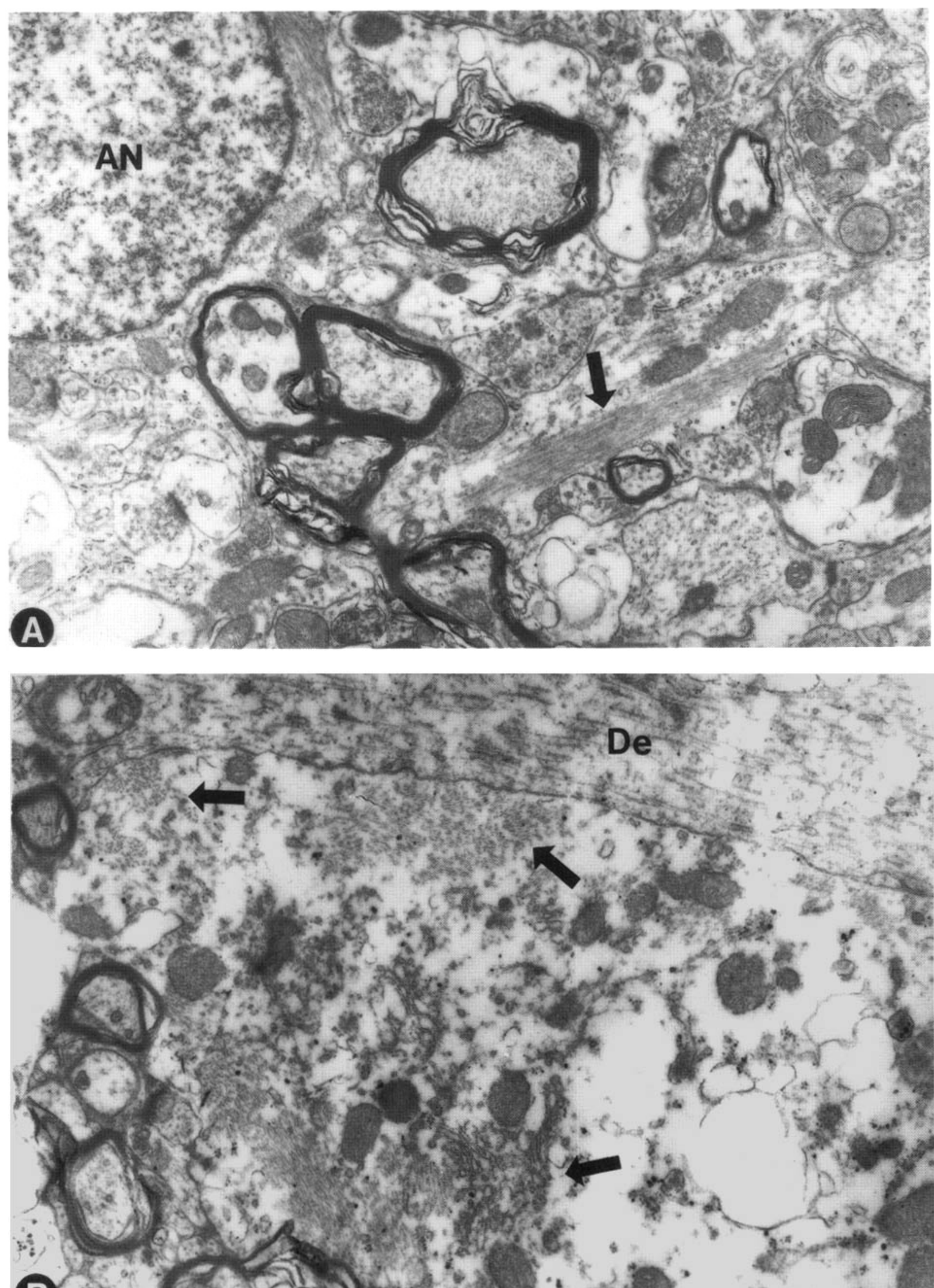

FIG. 43-3. Lumbar spinal cord. (**A**) Control animal shows normal appearance of neuropil. An astrocytic process exhibits a bundle of glial filaments (*arrow*); *AN*, astrocytic nucleus. (**B**) The center of the picture is occupied by an extremely swollen astrocytic process with dispersed glial filaments (*arrows*) and scattered membranous structures. Figure A: ×12,150; Figure B: ×18,750. [From Eng et al. (1989), with permission.]

new tools have permitted the study of molecular mechanisms of GFAP synthesis and degradation, phosphorylation/dephosphorylation, and glial filament assembly.

What are the consequences of astrocyte activation and induction of GFAP synthesis in disease and central nervous system injury? Is this a beneficial or detrimental response to central nervous system injury? How can one modulate astrogliosis to promote healing and functional recovery of damaged neuronal pathways? Our working hypothesis is that control of astrocyte activation and astrogliosis may be linked to GFAP synthesis. For example, inhibition of GFAP synthesis immediately following injury may delay astrogliosis and allow neurons time to regenerate and oligodendrocytes time to proliferate and remyelinate. In addition, activation of GFAP synthesis in astrocyte tumors may induce terminal differentiation and mitotic arrest. Our investigations of the biological function of GFAP employ a correlated morphological and biochemical approach to understanding gliosis. We have developed an *in vitro* model of astrogliosis in which astrocyte cultures are mechanically damaged (Yu et al., 1993). Current molecular biological techniques allow us to modulate GFAP synthesis in this injury model. GFAP can be detected by quantitative polymerase chain reaction (Galea and Feinstein, 1992). A quantitative polymerase chain reaction method has been developed which permits the identification of rare mRNAs and the quantitation of small and large changes following activation (Murphy et al., 1993). We have utilized antisense GFAP oligonucleotides to inhibit its synthesis in response to cAMP in normal (Yu et al., 1991) and injured astrocyte cultures (Yu et al., 1993). When specific GFAP enhancer sequences are identified, retroviral antisense constructs could be synthesized and might be used to inhibit GFAP transcription. With the *in vitro* mechanical injury model, we can systematically test hormones, growth factors, and other chemical mediators that promote or inhibit astrogliosis. Additional factors include mitogens, neurotransmitters, neurotoxic chemicals, extracts of degenerating neural tissue, and adhesion molecules as well as phorbol esters and other activators of protein kinases. New information obtained from *in vitro* experiments can be tested with *in vivo* models. With this information, we may be able to identify the cellular source of each factor and whether each response is beneficial or detrimental to central nervous system regeneration. This may allow us to design specific treatments to promote regeneration and function in the injured central nervous system. These treatments could employ antisense oligonucleotides, antibodies, growth factors, enzymes, pharmaceutical drugs, and gene therapies.

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IX Glia as Part of the Immune System

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44 Microglia and neuronal dysfunction

DANA GIULIAN

Microglia are a class of mononuclear phagocytes intrinsic to the central nervous system and are the principal immune effector elements of the brain (Giulian, 1987). Although microglia do not cause disease, they react to it and ultimately help to determine the pattern and degree of central nervous system recovery of function (Giulian, 1992b) through the secretion of bioactive agents including cytotoxins (Giulian, 1990). These cells, moreover, are closely associated with such diverse clinical problems as Alzheimer's disease, trauma, stroke, epilepsy, and acquired immunodeficiency syndrome (AIDS) (Giulian, 1992a). As argued here, future strategies to treat neurologic disorders will need to mitigate microglial disruption of neuronal function.

HISTORY

Rio Hortega (1932) described microglia as argentophilic cells with two or more "wavy, branched processes beset with spines." These ramified microglia were thought to make up at least 20% of the total glial population in normal, mature central nervous system. Further investigation showed that "ameboid" microglia emerged during embryonic development with such macrophagic properties as migratory capacity and phagocytosis. During central nervous system injury, ramified microglia retracted processes, became more ameboid-like, and transformed into a third form, the reactive microglia (Figure 44-1).

What is the origin of microglia? This question remains unanswered in part because of the lack of cell-specific markers suitable for study of early embryonic development (see Chapters 5 and 10). Rio Hortega (1932) felt that microglia arose from the mesoderm, first appearing as "roundish cells with pseudopodia." At present, most investigators agree that microglia arise during early embryonic development from blood precursor elements and later proliferate *in situ*. The bone-marrow chimera studies of Hickey and Kimura (1988) also indicate that perivascular central nervous system mononuclear phagocytes, but not parenchymal microglia, are exchanged with blood monocytes throughout normal adult life. In pathological situations, the origin of activated brain mononuclear phagocytes becomes more complicated. Invading blood-borne macrophages as well as reactive microglia were found after penetrating wounds (Giulian et al., 1989) or at sites of inflammatory-mediated demyelination (Hickey, 1991a). By contrast, microglia, but not invading macrophages, reacted to axotomy of the facial nucleus (Graeber et al., 1988). As noted by Rio Hortega, reactive microglia eventually take part in every type of inflammatory or necrotizing process in the brain.

MICROGLIA AS A DISTINCT CLASS OF MONONUCLEAR PHAGOCYTES

In vitro studies have been used to compare ameboid microglia with such other classes of mononuclear phagocytes as peritoneal macrophages, splenic macrophages, blood monocytes, and bone marrow progenitor cells (promonoblasts and monoblasts) (Metcalf, 1984). When grown under identical conditions in chemically defined culture medium, all mononuclear phagocytes possessed scavenger receptors that could be identified using a fluorescently labeled acetylated low-density lipoprotein (ac-LDL) (Figure 44-2). Microglia and macrophage were active phagocytes with very little response noted in marrow cells. Ameboid microglia and macrophages displayed chemotaxis. Spontaneous proliferation was noted for microglia, but not macrophages or monocytes; marrow cells would proliferate but only in the presence of growth factors. Ameboid microglia developed long, thin projections (up to several hundred microns in length), which resembled the branches of ramified microglia in tissue (Figure 44-3), while neither macrophages (short, stubby processes, if any) nor marrow cells (round or oval) displayed similar patterns of morphologic differentiation.

In another line of investigation, Kettenmann and coworkers (1991) have shown that membrane channel properties in microglia can be distinguished from

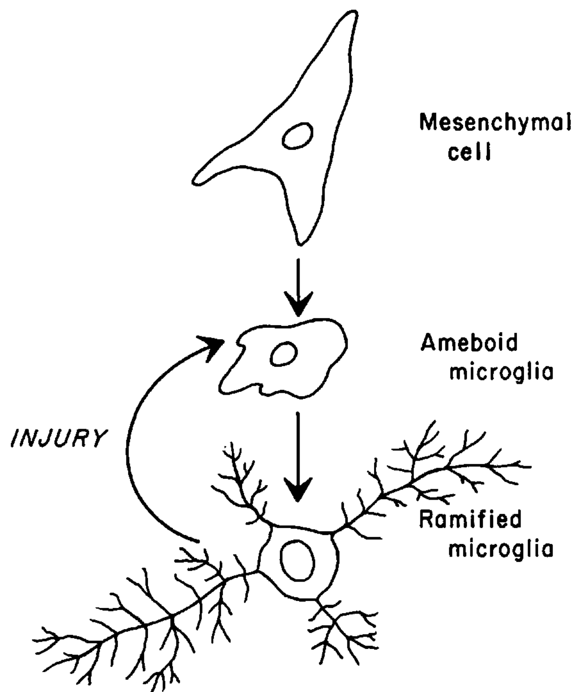
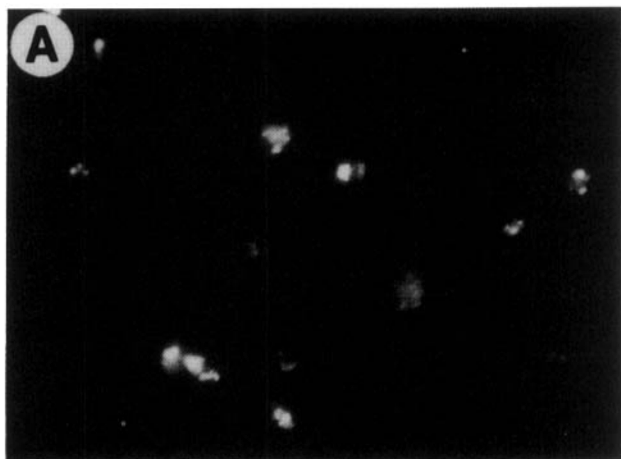


FIG. 44-1. The current view of microglial development. During early embryogenesis, mesenchymal cells migrate into the central nervous system. The earliest form of microglia are ameboid cells that show such macrophage properties as phagocytosis, release of cytokines, and scavenger receptors. During the postnatal period, microglia undergo differentiation to become ramified cells. These process-bearing cells lack macrophage properties and have no known function. However, at times of injury reactivation of the quiescent ramified cells occurs with retraction of processes and reappearance of macrophage functions.



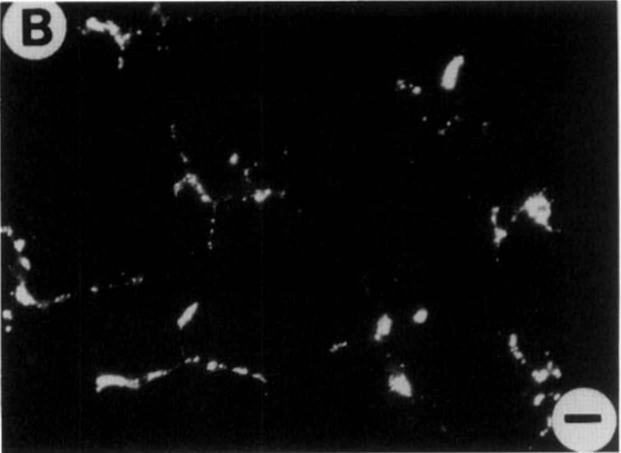


FIG. 44-2. Fluorescence photomicrographs showing cultures of rat peritoneal (A) macrophages or (B) microglia after 30 days *in vitro*. In chemically defined medium, macrophages take on a condensed, ameboid form with short, stubby processes. In contrast, microglia project thin processes that extend over hundreds of microns. The process-bearing microglia eventually lose macrophage properties and resemble ramified microglia found in tissues. Cells are labeled with a fluorescent probe for the ac-LDL receptor. Bar = 20 μm.

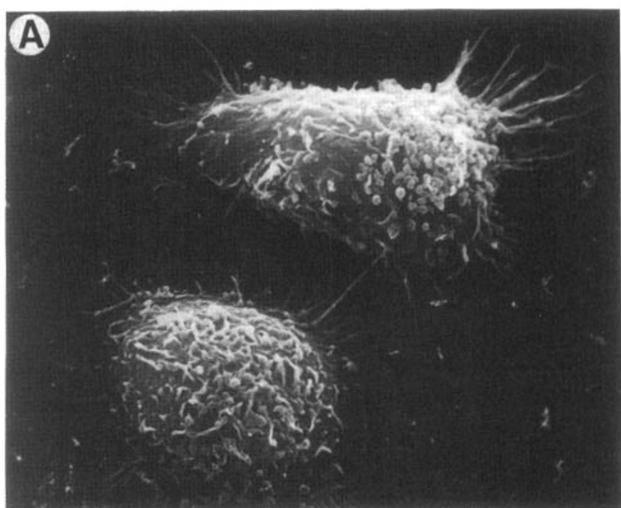
those in macrophages and blood monocytes. Using patch-clamp techniques, they found both ramified and ameboid microglia to contain large inward-directed rectifying currents dependent on K^+ (as indicated by reversal potentials and sensitivity to channel blockers). In contrast, macrophages displayed both a large outward rectifier current and a small inward one, while a subpopulation of bone marrow precursor cells had an inward-directed rectifying currents similar to that of microglia (Banati et al., 1991).

Present evidence, therefore, suggests microglia to be a distinctive class of mononuclear phagocytes that persist within the central nervous system after the perinatal period. In the ameboid form, microglia showed some properties common to tissue macrophages (surface markers, production of cytokines and cytotoxins) but displayed other features associated with bone marrow progenitor cells (proliferative capacity, response to colony-stimulating factors, and similarities in membrane current properties). It seems that these brain glia express a wide spectrum of features, ranging from those found in the more primitive as well as in the highly differentiated forms of mononuclear phagocytes. The overall pattern suggests a unique set of qualities that may have arisen during adaptation to the central nervous system (Giulian, 1992b).

REGULATORS OF MICROGLIAL REACTIVITY

Microglia found in the mature central nervous system are quiescent, ramified cells with no recognized function (Rio Hortega, 1932; Giulian, 1987; Giulian, 1992). Within days after central nervous system insult, ramified microglia expressed scavenger recep-

tors, retracted processes, migrated to areas of tissue debris, and engaged in vigorous phagocytosis. Membrane receptor densities often increased very rapidly and were among the first changes seen in reactive cells (Giulian et al., 1989). For example, microglia abutting damaged neurons displayed complement receptors within hours after axotomy of the facial nerve (Graeber et al., 1988). This precise pattern of cell activation suggested the early presence of a short-range signal (perhaps an unstable soluble factor or a contact-mediated event) to rouse close neighbors. In general, the range of microglial responses to central nervous system damage varied widely, with some cells showing only surface membrane changes or altered morphology, while others displayed full-blown phagocytic and secretory activities. For example, rod cells and lamellar cells (altered shapes) were associated with chronic infections such as rabies or tertiary syphilis, while rapidly appearing fat granule cells (active phagocytes) engulfed necrotic tissue (Rio Hortega and Penfield, 1927). Although signaling mechanisms doubtlessly vary with neuropathic conditions, the activation of microglia is a response common to all inflammatory and necrotizing processes in the brain. Thus, strategies to block microglial activation may prove effective in suppressing central nervous system inflammation.



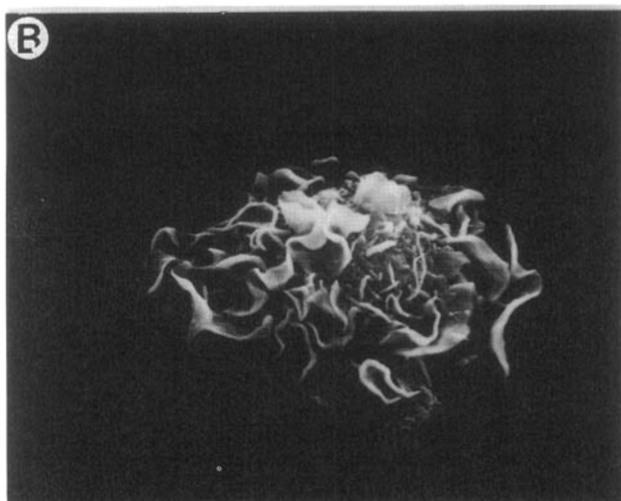


FIG. 44-3. (**A**) Cultured ameboid microglia have a surface morphology that can be distinguished from that of (**B**) tissue macrophages, using scanning electron microscopy. The characteristic spiny projections of microglia remind one of comments from Rio Hortega. [From Giulian and Baker (1986), with permission.]

What specific signals drive microglia to a reactive state? Rio Hortega (1932) has suggested that neuronal debris, blood clots, and dead cells are among the most potent signals to elicit reactive microgliosis. In culture systems, phagocytic signals (yeast wall particles or zymosan; fixed bacteria such as *Staphylococcus aureus*), cytokines, including interferon γ, and a variety of other immunoactivators (lectins, lipopolysaccharides, and phorbel esters) as scavenger receptor ligands and gp120, the coat glycoprotein of human immunodeficiency virus-1, have been used to elicit microglial secretion, changes in shape, appearance of surface markers, or chemotaxis responses (Giulian and Baker, 1986; Giulian, 1987; Giulian et al., 1991; Giulian et al., 1993b) (Table 44-1).

Immunomodulators have also been implicated as microglial activators *in vivo*. Hickey (1991b), for example, finds that systemic injections of tumor necrosis factor alpha (TNFα) elicit microglial major histocompatibility complex (MHC) class II receptors. Perhaps systemic immune responses, which generated cytokine release, initiate a reactive state within the brain. Granulocyte macrophage colony-stimulating factor (GM-CSF) has been found to expand microglial populations after infusion into brain tissues

TABLE 44-1. *Signals That Activate Microglia in Culture*[a]

Induction of cell proliferation
Brain-derived proteins
Granulocyte macrophage colony-stimulating factors
Multipotential colony-stimulating factors
Stimulation of cytokine release
Lipopolysaccharide
Phagocytic signals
Stimulation of neurotoxin release
Zymosan A
gp120 Viral protein
Scavenger receptor ligands

[a] *In vitro* studies show that a variety of agents promote microglial proliferation or secretory activity. Lipopolysaccharides stimulate release of such cytokines as interleukin-1 and tumor necrosis factor but not of neurotoxins. In contrast, the viral protein gp120 elicits neuron-killing factors but not cytokine secretion. These differential effects of activators suggest that cytokine and cytotoxin secretory mechanisms are independently regulated and point to discrete events that govern particular immune responses in the central nervous system.

(Giulian and Ingeman, 1988), and a GM-CSF-like factor has been recovered from injured brain (Giulian et al., 1991). Microglial sensitivity to immunoregulatory signals represents, therefore, an important link between immune and neural tissues.

MICROGLIA, ASTROGLIA, AND NEURONAL INJURY

Mononuclear phagocytes secrete cytotoxic factors (Adams, 1980; Khoo et al., 1981; Colton and Gilbert, 1987), which can be classified as short-lived (i.e., nitric oxide, hydrogen peroxide, or superoxide anion) or long-lived cell poisons (cytokines and enzymes; Table 44-2). Because cytotoxins have been implicated in various neurologic disorders, it was likely that brain inflammatory cells were important sources of these neuron-killing factors (Giulian, 1990; Giulian et al., 1990). In cell culture, microglia from newborn brain secreted neuron-killing factors, while astroglia released neuronal growth factors (Figure 44-4). The astroglial factors were found to be large molecules (>10 kD) (Vaca and Wendt, 1992; Giulian et al., 1993a), and the secreted microglial factors were heat-stable toxins of low molecular mass (Table 44-3). The astroglia-derived growth factors were proteins, sensitive to proteases and heat. Although not further characterized, these proteins probably included such astroglia-derived growth factors as neurotrophin-3, brain-derived growth factor, and ciliary neurotrophic factor (Ferrara et al., 1988; Manthorpe et al., 1989; Stockli et al., 1989). The microglia-derived neuron-killing factors were toxic to chick ciliary neurons and rat hippocampal and spinal cord neurons, but not to brain glia (Giulian et al., 1993a). Phagocytic signals (zymosan, fixed *S. aureus*) or scavenger receptor ligands were required to drive microglia from newborn brain to discharge neurotoxins (Table 44-1). The fact that lipopolysaccharide, a commonly used stimulant for cytokine release, did not elicit significant neuron-killing activity (Giulian et al., 1993b) suggests separate control mechanisms for release of cytokines and cytotoxins.

TABLE 44-2. *Cytotoxic Molecules Secreted by Mononuclear Phagocytes*[a]

Large molecules (>10 kD)
Tumor necrosis factor
Complement
Interferons
Interleukins
Proteases
Lipases
Small molecules (<1 kD)
Superoxide anion
Hydrogen peroxide
Nitric oxide
Eicosanoids
Lipoxins
Uric acid

From Giulian (1993), with permission.
[a]Examples of molecules secreted by mononuclear phagocytes that have cytotoxic properties. Target cell sensitivities and conditions under which these factors act as poisons vary considerably. However, the large molecules tend to be proteins with long-lived activity, while the small molecules are nonproteinaceous with short-lived actions. The unidentified microglial neurotoxins, in contrast, are small molecules with long-lived action.

MICROGLIAL-DERIVED NEUROTOXINS

The neurotoxic activity recovered from mononuclear phagocytes was stable in storage at −80°C and resistant to boiling (Table 44-3). Although brain mononuclear phagocytes were known to produce free radical intermediates with cytotoxic actions (Table 44-2), such highly reactive molecules would be heat-sensitive, and, therefore, not be responsible for the neuron-killing effects observed here. The inflammatory cell-derived neurotoxic activity was also resistant to proteinase K (100 μg/ml for 60 minutes at 37°C), ruling out protein cytokines and large peptides as toxic agents. Gel filtration chromatography showed that neuron-killing activities from all sources were small molecules, <500 Daltons.

Excitatory amino acids and their metabolites (as small, heat-stable, protease-insensitive agents) can serve as neurotoxins in the mammalian brain by acting through the *N*-methyl-D-aspartate (NMDA) class of glutamate receptor (Dingledine, 1986; Honore, 1989). The selective NMDA antagonist 2-amino-5-phosphopentanoic acid (AP5) and NMDA-receptor ion channel blocker MK801 protected against the neurotoxicity secreted by mononuclear phagocytes, while the antagonists to non-NMDA-type glutamate receptors [2-amino-3-phosphonopropanoic acid (AP3), 6-cyano-7-nitroquinoxoline-2,3-dione (CNQX) and gamma-D-glutamylaminomethylsulfonic acid (GAMS)] did not do so (Table 44-3). The microglia-derived neurotoxins could be separated from such NMDA agonists as excitatory amino acids, quinolinic acid, and polyamines by ion exchange chromatography and reverse-phase high-performance liquid chromatography (Giulian et al., 1993a). In addition, partially purified neurotoxins showed very low concentrations of the excitatory amino acids glutamate and aspartate (<100 pM), or quinolinic acid (<2 nM), which were all below the toxic range for cultured ciliary neurons (Giulian et al., 1993a). Microglia, therefore, released a class of small, stable neuron poisons with actions mediated

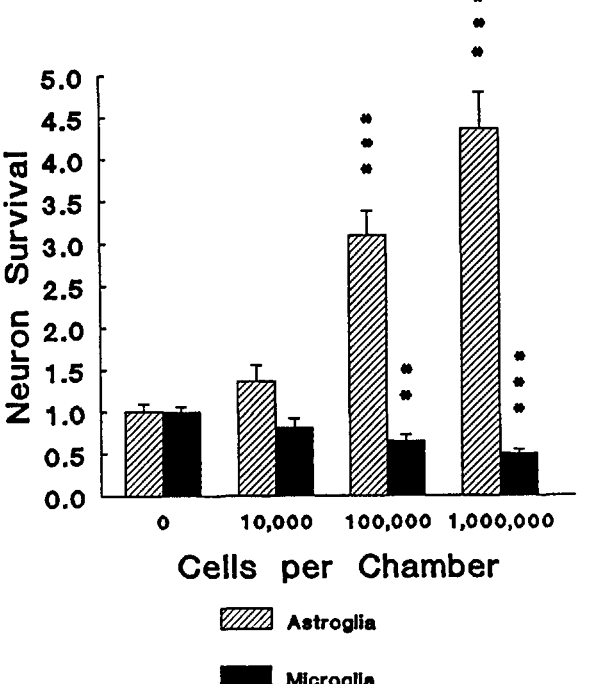


FIG. 44-4. Microglia secrete neurotoxins and astroglia release neuron survival-promoting factors. Isolated DiI-ac-LDL(+) microglia (>98% homogeneity) or GFAP(+) astroglia (>99% homogeneous population) were seeded at the densities indicated and placed in MilliCell-CM chambers. The chambers were then placed into wells (12 well plates; 3.8 cm^2 per well) and cocultured for 48 hours with approximately 8000 chick ciliary neurons per well grown on poly-L-lysine-coated glass coverslips. Both microglia and astroglia were incubated with zymosan A particles. Viable neurons were identified by phase contrast microscopy at ×200 magnification. Neuron survival scores (neurons per field in treated group/neurons per field in the untreated control group) are presented as mean values ± standard error. Each value was obtained from 18 fields per coverslip, using at least 4 coverslips per group. Zymosan A particles placed in the MilliCell-CM chambers alone did not affect neuronal survival. Significant differences among controls and microglia or astroglia cocultures are noted at 100,000 and at 1,000,000 cell concentrations using Student's *t* test ($^{**}p < .001$; $^{***}p < .0001$) with a confidence level for 6 comparisons estimated at $p < .008$ using the Bonferroni method.

by NMDA receptors that could be distinguished from known brain-derived NMDA agonists.

Since our initial observations, the concept of activated microglia as a source of neurotoxic factors has been confirmed by other investigators in a number of different settings. During the past few years, Pulliam et al. (1991) and Piani et al. (1991) have described microglial release of neurotoxins in different cell culture systems. There has also been extensive work on microglial secretion of free radicals in the laboratories of Colton and Gilbert (1987) and Thery et al. (1991). More recently, *in vivo* experiments from Thanos et al. (1993) have suggested mononuclear phagocyte-mediated damage of neurons. Thus, the release of microglia-derived cytotoxins appears to be a common pathway in many neuropathological processes.

MICROGLIA AND TRAUMATIC BRAIN INJURY

Cell culture experiments demonstrated that astroglia or microglia could regulate neuron survival through the actions of secretion products. By combining glial secretion products, it was apparent that increasing amounts of astroglial-conditioned medium attenuated the toxic effects of microglia (Figure 44-5). This *in vitro* competition between glial populations suggested that opposing secretory mechanisms might influence *in vivo* tissue viability. To test such a hypothesis, different types of central nervous system injury were monitored for tissue production of neuron-promoting or neuron-killing factors.

Penetrating trauma to the rat neocortex elicits an inflammatory response marked by the appearance of both reactive microglia and invading macrophages which bear scavenger receptors within 5 to 8 hours after injury (Giulian et al., 1989). The greatest number of these inflammatory cells appear at 2 days after injury (Figure 44-6A; from Giulian et al., 1993a). To monitor the presence of neurotoxins during the course of central nervous system injury, punch microdissections of wound sites (recovered 12 hours to 7 days after trauma) were incubated in culture medium and tested for effects upon ciliary neuron assay. Although little cytotoxic activity was detected before or after the peak of inflammation, nearly 50% of neurons were destroyed when incubated with tissue-derived factors isolated 2 days after trauma (Figure 44-6C). These neurotoxic factors were of low molecular mass (<1 kD), were blocked by the NMDA antagonist AP5, and appeared identical in action to those toxins derived from isolated microglia (Table 44-2). Further study showed that media conditioned by injured tissue also contained neuron-promoting factors (Figure 44-6B) as noted by other investigators (Needels et al., 1987). These factors were trypsin-sensitive growth-promoting molecules (>10 kD), present in undamaged tissues (Table 44-3), and similar to proteins derived from cultured astroglia (Giulian et al., 1993a; see also Chapter 30).

MICROGLIA AND CENTRAL NERVOUS SYSTEM ISCHEMIA

Ischemia to the central nervous system can produce a delayed decline in neurologic function that occurs

TABLE 44-3. *Cell Types and Release of Neuron Growth-Regulating Factors*[a]

	Microglia	Astroglia	Reactive Phagocytes
Promotes neuron survival	No	Yes	No
Molecular mass	<500 Daltons	>10 kD	<500 Daltons
Heat-sensitive	No	Yes	No
Protease-sensitive	No	Yes	No
NMDA receptor blockade AP5	Yes	No	Yes
MK801	Yes	No	Yes
Non-NMDA receptor blockage			
GAMS	No	No	No
CNQX	No	No	No
Calcium L-channel blockage			
Verapamil	No	Yes	No
Nifedipine	No	Yes	No

From Giulian et al. (1993a), with permission.
NMDA, *N*-methyl-D-aspartate; AP5, 2-amino-5-phosphopentanoic acid; GAMS, gamma-D-glutamylaminomethylsulfonic acid; CNQX, 6-cyano-7-nitroquinoxoline-2,3-diones.
[a]Properties of molecules released by microglia and astroglia isolated from neonatal brain or from reactive mononuclear phagocytes isolated from adult brain damaged by ischemia. Conditioned media were heated by boiling for 10 minutes at pH 7.4. Protease treatments for factors from microglia and reactive cells involved proteinase K (1 μg/ml) for 2 hours at 37°C and stopped by ultrafiltration. Astroglial factors were incubated with trypsin (1 μg/ml) for 2 hours at 37°C and then inhibited with soybean trypsin inhibitor (2 μg/ml). Toxic activities from microglia and reactive phagocytes were associated with small, stable, nonproteinaceous molecules mediated through NMDA receptors. In contrast, astroglia released growth-promoting proteins whose actions involve calcium L-channels.

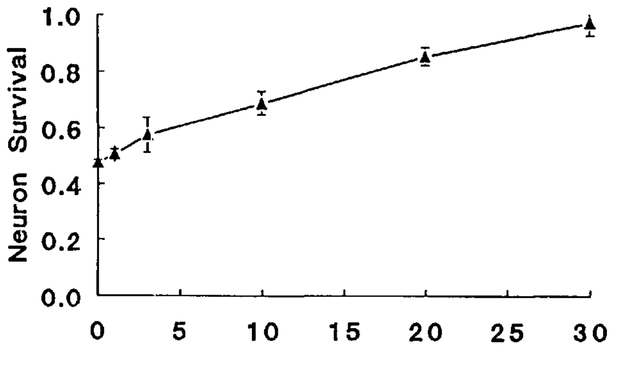
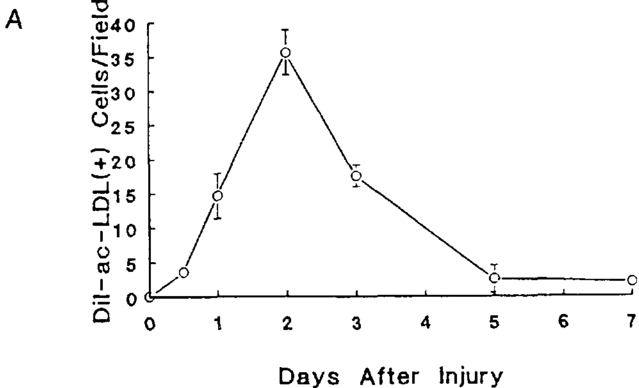
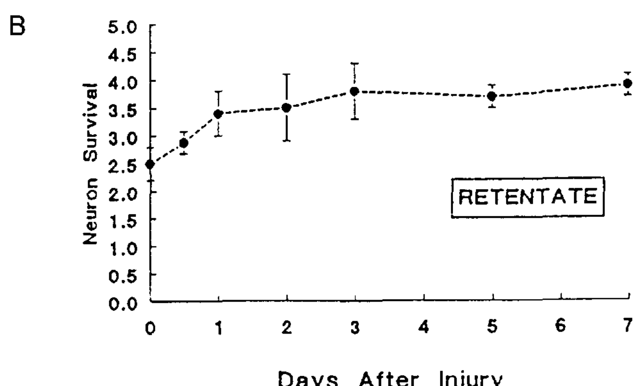


FIG. 44-5. Opposing effects of glial secretion products upon neuronal survival. Ciliary neurons were treated with microglial toxic activity (20% conditioned medium by volume from 10^6 cells activated with zymosan for 48 hours) in the presence of growth factors found in equal volume of medium conditioned by astroglia (from 10^6 cells incubated with zymosan). As shown, astroglial factors attenuated the microglial toxins. Such observations suggest that different glial populations have competing actions upon the survival of neurons. Neuron survival scores (neurons per field in treated group/neurons per field in the untreated control group) are presented as mean values ± standard error. Significant differences were noted between astroglia and other treatment groups using the Student's *t* test ($**p < .001$).

within the first several days after injury. Transient occlusion of the rabbit abdominal aorta (20-minute occlusion) caused ischemic damage in the spinal cord with a striking inflammatory response seen within the first 24 hours (Giulian and Robertson, 1990), particularly at sites of neuronal damage in the lower lumbar cord. Serial sections of spinal cord (extending from the lower thoracic to upper sacral) were placed in culture dishes containing neurons and after a 24-hour coculture period, punch microdissections of grey matter were taken of the tissue sections to monitor the presence of DiI-ac-LDL(+) mononuclear phagocytes. Spinal cord tissue from a normal rabbit contained astroglia with glial fibrillary acidic protein (GFAP), lacked DiI-ac-LDL(+) mononuclear phagocytes, and released growth-promoting factors that increased neuronal survival by 2- to 3-fold (Figure 44-7A). In general, the spinal cord damaged by ischemia was less supportive of neuronal growth with some contiguous sections of tissue showing significant release of neuron-killing activity (Giulian and Vaca, 1993) (Figure 44-7B). Importantly, tissues taken from a region of the lower lumbar cord both produced a striking reduction in neuron survival and





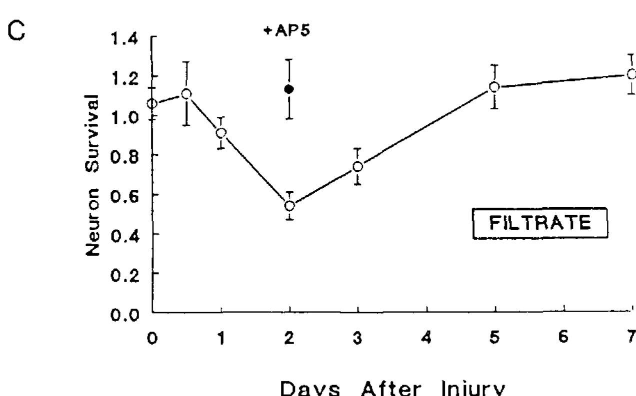
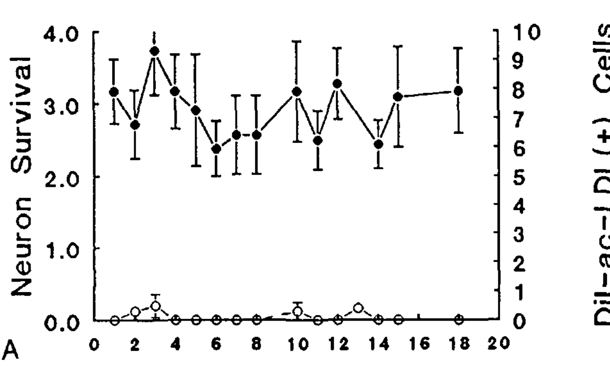


FIG. 44-6. Traumatic injury to the neocortex of adult rat elicits tissue production of neurotoxins. (A) Time course of inflammation at the site of a penetrating wound placed 1.0 mm from the surface of the brain. The peak number of DiI-ac-LDL(+) mononuclear phagocytes occurs at about 2 days after injury. Data represent mean values ± standard error taken from at least 10 fields per punch microdissection when viewed at ×200 magnification. Ultrafiltration of media conditioned by wound site tissues showed the presence of both (B) neuron-promoting factors (retentate >3 kD) and (C) neurotoxins (filtrate <3 kD). The neurotoxic effects for tissue-derived toxins were blocked by 10 μM AP5 as noted for microglia-derived cell poisons ($p < .01$ by Student's t test). The greatest concentrations of neurotoxic activity were recovered at 2 days after injury or the peak of the inflammatory response. Values are expressed as mean neuronal survival score ± standard error with conditioned media tested at concentrations of 20% by volume.

held the largest population of DiI-ac-LDL(+) cells (Figure 44-7B). Tissues most heavily infiltrated with activated mononuclear phagocytes were, therefore, the greatest source of neuron-killing activity.

To confirm the cellular sources of the neurotoxic activity within damaged spinal cord, central nervous system mononuclear phagocytes were isolated by selective adherence to plastic. More than 90% of cells



Ischemic Injury

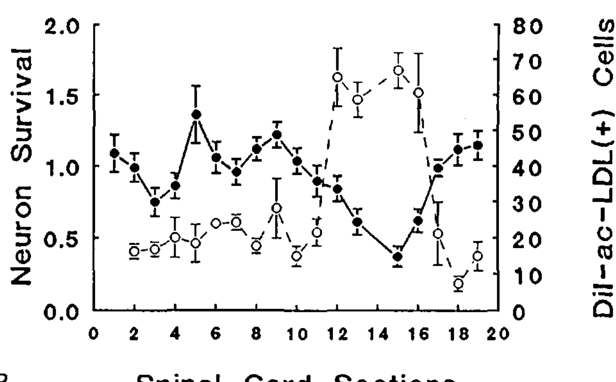


FIG. 44-7. The spinal cord of rabbit releases neurotoxins when damaged by ischemia. (A) Serial sections (1 cm thick) obtained from the lumbar spinal cord of a normal rabbit were coincubated with ciliary neuron cultures for 24 hours. In the presence of normal rabbit tissue, neuron survival was increased by about threefold. Undamaged tissue showed no evidence of reactive mononuclear phagocytes as determined by labeling with DiI-ac-LDL. (B) In contrast, serial sections obtained 2 days after ischemic injury showed inflammatory cells throughout the lumbar spinal cord with heavily inflamed tissue noted in lower region of the cord. These same sections (sections 12 through 16) showed the greatest production of neuron-killing factors demonstrated by the decline in ciliary cell survival. These observations point to a close association between central nervous system inflammation and the release of neurotoxins. Assays were performed upon coded, randomized samples with values expressed as mean survival scores ± standard error. Mononuclear phagocytes in spinal cord sections were viable as demonstrated by the ability to endocytose DiI-ac-LDL.

recovered from rabbit spinal cord were DiI-ac-LDL(+) macrophagelike phagocytes laden with debris. These cultured cells released a neurotoxin of low molecular mass that was sensitive to AP5 blockade (Giulian and Vaca, 1993). In contrast, nonadherent, DiI-ac-LDL(−) cell populations (containing >75% GFAP(+) astroglia) produced factors promoting neuronal growth. These results further supported the idea that reactive microglia were a source of neuronal poisons, while neighboring astroglia sustained neuron survival.

IMMUNOSUPPRESSION AS TREATMENT FOR ISCHEMIC INJURY

Time course studies have suggested that reactive mononuclear phagocytes migrate to the site of injury and engulf debris prior to the release of cytotoxic factors (Figure 44-6; Giulian, 1992). Suppression of such initial cellular events, therefore, might offer some clinical benefit by preventing subsequent production of neuron poisons. Drugs, known to inhibit endocytosis, phagocytosis, and secretory function in mononuclear phagocytes have been tested as potential suppressants of central nervous system inflammation (Giulian et al., 1989; Giulian and Robertson, 1990). Chloroquine and colchicine, when given by intraperitoneal injection at 60 minutes after ischemic injury, reduced the numbers of these scavenger receptor bearing cells by about 75% at the site of infarction. A similar inhibition was noted when comparing factors released from 2-day-old infarcts with factors recovered from lesions 1 day after injury (prior to the appearance of most activated mononuclear phagocytes) or from drug-treated animals 2 days after injury. Tissues damaged by ischemia, but containing few inflammatory cells, released no measurable neurotoxic activity, whereas inflamed tissues released factors that killed neurons. Thus, in the absence of an inflammatory reaction, severely damaged neural tissues did not produce significant amounts of neurotoxic activity. However, animals that received chloroquine plus colchicine at the time of middle cerebral artery occlusion also showed no reduction in the volume of infarcted tissue. Thus, it was not likely that the direct tissue injury brought on by ischemia (which rapidly destroyed both neurons and glia at the site of infarction) was under immune control. Although quiescent brain mononuclear phagocytes did not effect neuronal sensitivity to acute ischemic insult, reactive phagocytes did influence neuronal survival, but at a later period.

Does immunosuppression alter functional recovery after injury? As noted earlier, a moderate degree of ischemia elicited a large number of reactive mononuclear phagocytes in the rabbit spinal cord. The close association between the appearance of activated mononuclear phagocytes and deterioration in hindlimb function suggested an immune-mediated loss of function (Giulian and Robertson, 1990). To test this hypothesis, chloroquine and colchicine were given at 6 hours after injury and continued for a total of 3 days. Transverse sections of the lumbar spinal cord showed that the gray matter of control animals have massive invasions of phagocytes whereas drug-treatment led to a substantial reduction of inflammation (Table 44-4). Importantly, far more surviving neurons were found in the anterior horn of animals receiving chloroquine and colchicine than in control animals (Table 44-4). Control animals typically have a decline in hindlimb function by 24 to 36 hours after injury. In contrast, chloroquine-colchicine-treated animals either maintained a high level of function or showed improvement from the baseline examination obtained prior to treatment (Table 44-4). This level of hindlimb strength remained stable for at least 7 days after injury as did bladder function. Thus, early control of central ner-

TABLE 44-4. *Effects of Immunosuppression After Ischemic Damage to Rabbit Spinal Cord*

	Control	Chloroquine /colchicine
Histologic findings		
Reactive mononuclear phagocytes (cells per field)	58 ± 6	5 ± 3
Motor neuron survival (%)	28 ± 6	77 ± 10

Following occlusion of the abdominal aorta for 20 minutes, rabbits were treated with chloroquine + colchicine (1.0 mg/kg and 0.2 mg/kg, respectively) per day for 3 days. The numbers of DiI-ac-LDL(+) mononuclear phagocytes found in gray matter of lumbar spinal cord at 3 days postinjury were significantly reduced by drug treatment when compared to untreated controls ($p<.001$ by Student's t test). Values are expressed as mean number of cells ± standard error per field (×200 magnification).

Reduction in the inflammatory response by chloroquine plus colchicine is correlated to an increase in motoneuron survival ($p<.005$ by Student's t test). Values are expressed as mean percent motor neuron survival (number of neurons in treated group/number of neurons in normal animals) ± standard error. Scoring used coded cresyl violet-stained sections (20 μm thick) of spinal cord at the level of L5. Normal animals showed 18.1 ± 0.5 large neurons per field of the anterior horn when viewed (×200 magnification).

	Control	Chloroquine /colchicine
Functional outcome		
Hopping behavior	0/6	4/6
Bladder control	1/6	5/6

Immunosuppression began at 6 hours after injury with one intravenous dose of 1 mg/kg chloroquine plus 2.5 mg/kg per day of chloroquine intramuscularly and 0.2 mg/kg per day colchicine intravenously. Identical daily doses of intramuscular chloroquine and intravenous colchicine were given for 3 days. Motor and bladder examinations were performed every 6 to 8 hours for 7 days.

From Giulian and Robertson (1990), with permission.

vous system inflammation improved long-term recovery of neurologic function.

MICROGLIA AND EPILEPSY

Although trauma, meningitis, and encephalitis have been associated with the onset of seizures, the mechanisms by which these pathologic conditions produced aberrant electrical discharges are unknown. One feature common to these diverse central nervous system injuries was the presence of brain inflammation. Since blood-borne invading macrophages and reactive microglia secreted a complex mixture of substances, including arachidonic acid metabolites, cytokines, amino acids, and cytotoxins, it seemed reasonable that inflammatory cells might also secrete factors that alter electrical activity within the central nervous system.

The hippocampus has a propensity for epileptiform activity (Alger, 1984) and can be a site of origin for complex partial seizures. Moreover, the cellular organization of the CA3 region in the hippocampus consists of many local circuits capable of synchronous bursting, the hallmark of epileptiform activity (Johnston et al., 1986). For these reasons, factors secreted by activated mononuclear phagocytes were examined for effects upon electrical activity in the CA3 region of the hippocampal slice preparation (Rutecki et al., 1985). Brief exposure to microglial or macrophagic secretion products induced a series of epileptiform discharges in tissue slices (D. Giulian and P. Rutecki, unpublished observations).

The observation that inflammatory cells release neuroexcitants opens a new area of investigation about immune-mediated mechanisms of seizure disorders. The data indicate that reactive mononuclear phagocytes secreted agents that both damaged central nervous system tissues as well as induced seizures. Moreover, preliminary study has shown that inflammatory cells excite neighboring neurons in such a way as to induce electrical activity that persisted long after resolution of the inflammation. Perhaps "immunokindling" is an important early event in types of epilepsy associated with infection or trauma. See also Chapter 62, this volume.

MICROGLIA AND ALZHEIMER'S DISEASE

Alzheimer's disease is a neurodegenerative dementia associated with loss of neurons, accumulation of paired helical filaments, and the deposit of amyloid in senile plaques and vessel walls (Terry, 1985). Although Bolsi (1927) long ago noted argentophilic reactive cells surrounding senile plaques, the significance of reactive microglia in Alzheimer's disease has only recently received much attention (Cras et al., 1990; Styren et al., 1990). Using the histocompatibility marker HLA-DR as an indicator of activation, McGeer et al. (1988) have described numerous reactive microglia within the corona of senile plaques. Moreover, Griffin et al. (1989) have found interleukin-1 production in microglia surrounding amyloid plaques. Although it seemed unlikely that microglial abnormalities were a primary cause of senile dementia, these cells could influence secondary events, which have been identified as important pathologic features of Alzheimer's disease. For example, contact between microglial membranes and amyloid deposits implied an intimate link to microglial function. Perhaps reactive cells provided a necessary element in the plaque-forming process. Alternatively, core material found in plaques of Alzheimer's disease has been noted to be very resistant to chemical attack (Nikaido et al., 1971) and could, therefore, subvert the debris-clearing capacities of microglia. In this way, the plaques could serve as stable irritants that hold the central nervous system in a chronic state of inflammation. Activated microglia might, in turn, influence the disease course by accelerating the rate of neuron death. While these issues remain to be clarified, it is clear that chronic brain inflammation might further impair cognitive abilities of patients with Alzheimer's disease. See also Chapter 68, this volume.

MICROGLIA AND AIDS

Acquired immunodeficiency syndrome produces a devastating effect upon the brain and spinal cord, with more than 70% of all patients showing loss of memory, paralysis, seizures, sensory deficits, or global dementia (McArthur, 1987). Human immunodeficiency virus-1 (HIV-1) has been isolated from the central nervous system of AIDS patients and identified in several classes of central nervous system mononuclear phagocytes, for example, microglia, macrophages, and multinucleated macrophage-like cells (Budka et al., 1989). At autopsy, the brains of AIDS patients with dementia showed cortical atrophy and loss of cortical neurons (Navia et al., 1986). Although direct viral infection of neurons in brain tissue has not been demonstrated by immunohistology or by *in situ* hybridization (Koenig et al., 1986; Wiley et al., 1986), recent ultrastructural study had

shown extensive neuronal damage (Wiley et al., 1991). Thus, neurologic disorders of AIDS appear to be an indirect effect of retrovirus infection and might be the result of neurotoxic factors.

Several specific types of neurotoxic mechanisms have been put forth to account for neuron death associated with HIV-1 infection. Brenneman et al. (1988) and Dreyer et al. (1990) have reported the viral coat glycoprotein gp120 to be a neuron poison. A second hypothesis, and the one favored by my laboratory (Giulian et al., 1990; Pulliam et al., 1991; Giulian et al., 1993c), has been that *inflammatory* cells infected with HIV-1 release neuron-killing factors. Alternatively, the neurotoxin quinolinic acid (Schwarcz et al., 1983) might be a toxic factor in AIDS dementia, since elevated quinolinic acid concentrations were found in the cerebrospinal fluid of patients suffering from AIDS encephalopathy (Heyes et al., 1989).

A coculture system was used to test whether HIV-1-infected cells release neurotoxic factors. Test cells, with or without HIV-1 infection, were placed in filtered chambers (pore size <0.4 μm) and then cocultured with target neurons grown atop glass coverslips. Using this approach, factors secreted by viral human cells could be examined for effects upon cultures of chick ciliary ganglion, rat embryonic spinal cord neurons, or rat embryonic hippocampal neurons (Giulian et al., 1990). The human CD4(+) cell lines that were tested had been constitutively infected with HIV-1 and included the lymphoid cell line H9, promonocytic cell line U937, and the monocytoid cell line THP-1. After 48 hours, neurons were quite robust-looking, both in control cultures or in cocultures involving uninfected CD4(+) cells. However, coculture with infected monocytoid cells brought about dramatic neuronal losses (Figure 44-8). This pattern of cell killing could also be observed for neurofilament(+) rat spinal cord neurons. In contrast, the lymphoid cell line H9 with or without HIV-1 infection did not produce neurotoxins. Since H9 cells released significant quantities of virions and viral proteins (as much as 10-fold higher than observed for media incubated with monocytoid cells), it appeared that HIV-1 or its constituents were not toxic to cultured neurons. Direct experiments confirmed that recombinant proteins from HIV-1, including the structural protein gag, enzymes such as HIV-1 protease and reverse transcriptase, or gp120, had no direct toxic effects upon chick ciliary neurons, rat spinal cord neurons, or rat hippocampal cells (Giulian et al., 1993c). To determine whether an indirect neuron-damaging mechanism was involved, human blood lymphocytes and monocytes, or the cell lines THP-1, H9, and U937, were incubated with recombinant HIV-1 proteins. Only gp120 stimulated monocytoid cells to elicit neurotoxic activity (Figure 44-9). There was no release of neuron-killing molecules from lymphocytes or H9 cells exposed to gp120 or the other recombinant proteins.

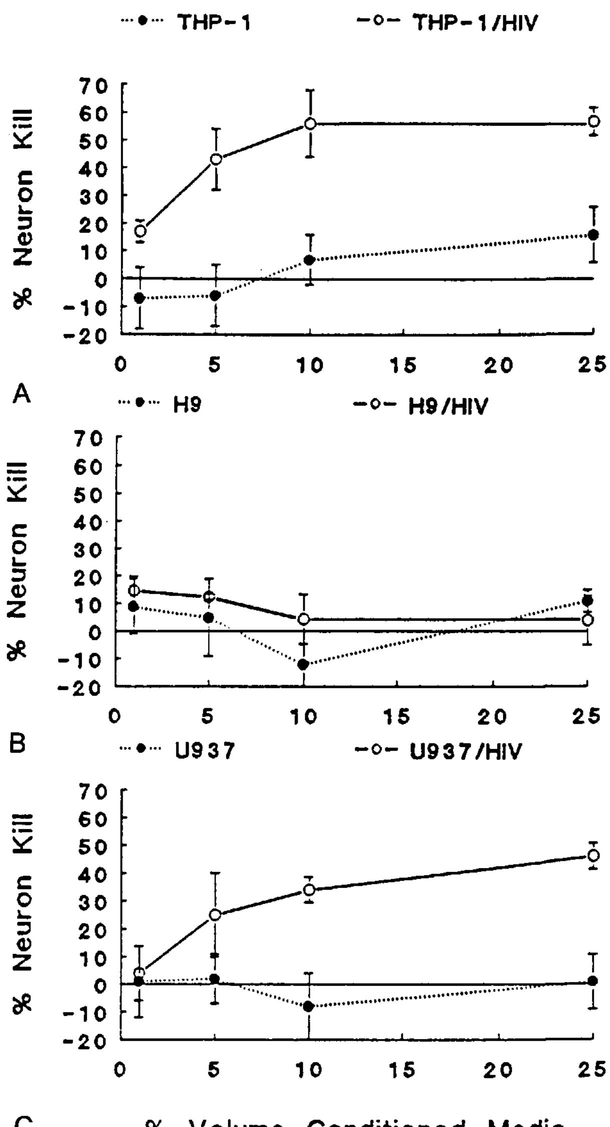


FIG. 44-8. The percent neuron kill for each experiment was expressed as mean number of neurons per field for a treated culture/mean number of neurons per field for untreated control cultures. Every value, expressed as mean% neuron kill + standard error, was obtained from at least three cultures. The uninfected (*open bars*) or HIV-infected (*filled bars*) cell lines were plated at the indicated densities in Millicell-CM chambers. The chambers were then transferred to 3.8 cm^2 wells, each containing 10,000 neurons and incubated for 48 hours prior to fixation. (A) THP-1 monocytoid cells. (B) H9 lymphoid cells. (C) U937 promonocytoid cells. [From Giulian et al. (1990), with permission.]

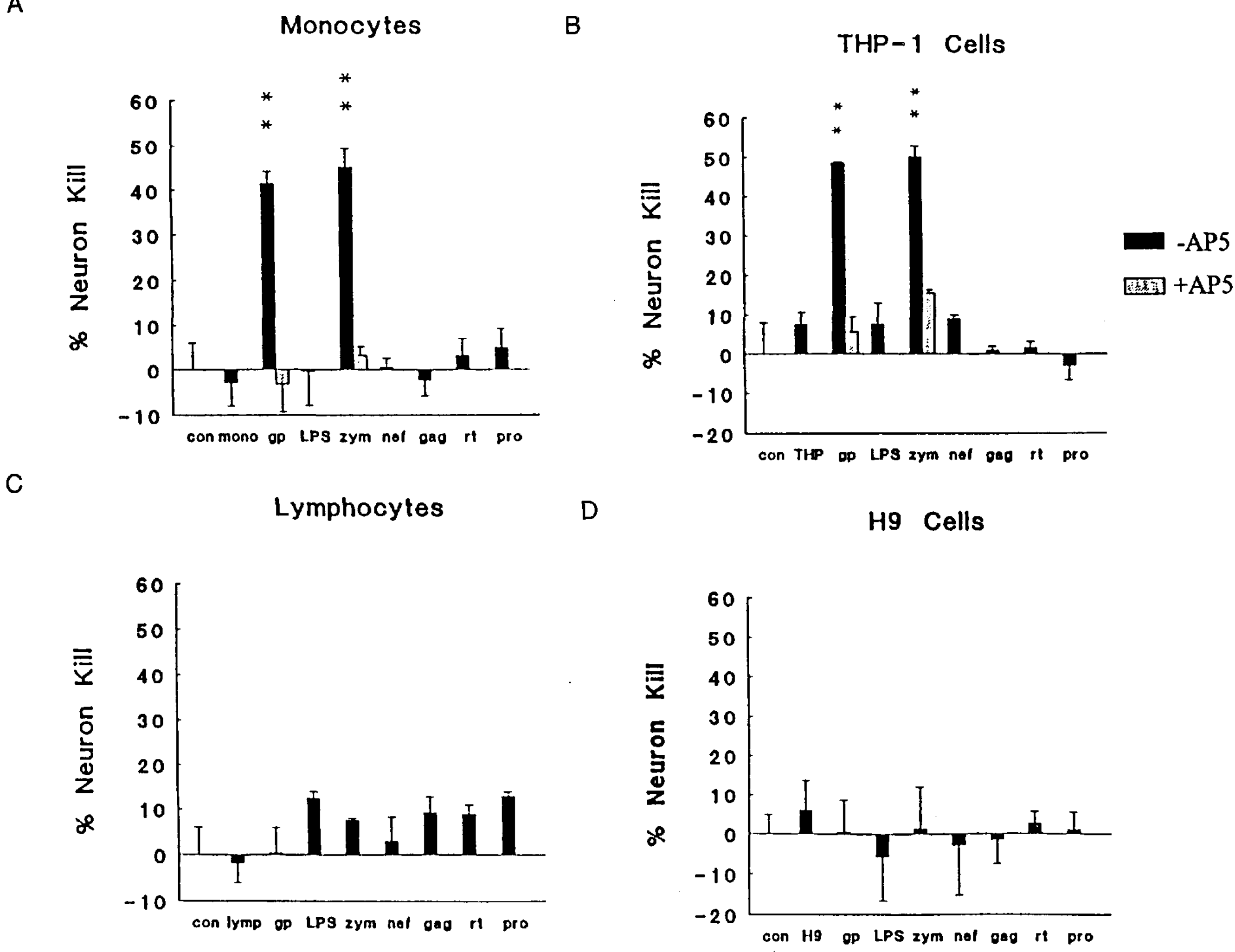


FIG. 44-9. Indirect toxicity of gp120 on ciliary neurons. (A) Isolated human blood monocytes, (B) the monocytoid human cell line THP-1, (C) isolated human blood lymphocytes, or (D) the lymphoid human cell line H-9 were incubated for 48 hours in the presence of 10 nM of various recombinant-derived HIV-1 proteins. Media from the treated cells were then assayed at a final concentration of 20% volume on ciliary neurons for the presence of neurotoxicity. As shown, only gp120 (gp)-stimulated monocytes (Figure A) or THP-1 cells (Figure B) released factors that were toxic to ciliary neurons. These effects were significantly elevated above the untreated control cultures (*con*) using the Student's t test ($^{**}p < .001$) with a confidence level of $p < .006$ by the Bonferroni method for 8 comparisons. Importantly, this toxic activity could be attenuated by 10 μM of the NMDA antagonist AP5 (Figures A and B). Zymosan A particles (*zym*), but not LPS, also elicit neurotoxin release in the monocytoid cells (Figures A and B) but not in lymphoid cells (Figures C and D). Media from unstimulated monocytes (*mono*), THP-1 cells (*THP*), lymphocytes (*lymp*), or H-9 cells (*H9*) did not produce neuron-killing factors (Figures A–D). HIV-1 protease (*pro*), reverse transcriptase (*rt*), p25/24 gag (*gag*). [From Giulian et al. (1993c), with permission.]

As sources of neurotoxic factors, brain mononuclear phagocytes, therefore, might create a persistantly disruptive influence upon neurologic function in HIV-1-infected individuals. The neurotoxins released by HIV-1-infected or gp120-stimulated monocytoid cells consisted of small molecules, which were heat-stable, protease-resistant, and acted via the NMDA receptor. As noted for reactive microglia, the HIV-1-associated neurotoxic activity was not quinolinic acid or any of the excitatory amino acids. See also Chapter 67, this volume.

CONCLUSIONS

Although reactive gliosis has long been thought to impair recovery of neurologic function (Aguayo et al., 1981; Reier, 1983), it remains unclear whether astroglia or microglia were responsible for such impairment. As noted by other laboratories, astroglia produced proteins which stimulate the outgrowth and survival of neurons in culture (Manthorpe et al., 1986; Ferrara et al., 1988; Manthorpe et al., 1989; Vaca and Wendt, 1992). Such astroglia-derived fac-

tors were found within normal neural tissues and within damaged tissues neighboring sites of trauma (Needels et al., 1987) and might include ciliary neurotrophic factor, brain-derived growth factor, and neurotrophic factor-3 all which are produced by astroglia (Vaca and Wendt, 1992). In contrast, brain inflammatory cells were a source both of short-lived free radicals which participated in contact-mediated killing of neurons and of long-lived neurotoxic factors that appeared after traumatic and ischemic injury. The release of long-lived inflammatory cytotoxins was a delayed process and not detected until a peak of cellular reactivity was reached, often by the second day after injury. The unopposed effects of neurotoxins from microglia and macrophages (see also Chapter 29) during the acute phase of inflammation might limit survival or function of neurons. This neuron-killing action could, in turn, be balanced by growth factors released from astroglia at a later phase of wound repair so that recovery of neuronal function would depend to a degree upon the location and numbers of reactive glia. Destruction of neurons neighboring sites of injury might be an important mechanism by which reactive microglia quell the spread of neurologic dysfunction (Giulian, 1992). As noted by Rio-Hortega (1932), necrotic tissue or hemorrhage elicit striking brain inflammatory reactions. As a potent stimuli for reactive microgliosis, tissue debris might continue to drive local production of neuron-killing factors in response to a variety of central nervous system injuries (Giulian, 1992) and, therefore, influence neuronal survival well beyond the period of initial tissue insult.

Microglia-derived neurotoxins were heat-stable protease-resistant molecules <500 Daltons with actions blocked by *N*-methyl-D-aspartate (NMDA) receptor antagonists. These molecules were distinguished from free radical intermediates and separated from excitatory amino acids and from the NMDA receptor-mediated toxin quinolinic acid by ion exchange and reverse-phase chromatography. In addition, the neurotoxic activities released from microglia or macrophages were small molecules with long-lived action and could be distinguished from a number of secreted cell poisons. Purification of microglia-derived neurotoxins will be necessary to uncover their precise mechanisms of action and to determine their relationships to NMDA agonists.

Five lines of evidence now support the idea that microglia-derived neurotoxins have an important role during neural tissue damage (Giulian, 1992).

1. Neurotoxins were only detected in tissues heavily infiltrated with reactive microglia or macrophages. The levels of neurotoxic activity found in central nervous system injured by either trauma or ischemia correlated to the number of mononuclear phagocytes at the lesion sites. Importantly, no toxic activity was detected in neighboring noninflamed tissues or in normal animals.
2. Drugs that reduced inflammatory cell numbers also reduced the amount of neuron-killing activity released by damaged tissues.
3. Active mononuclear phagocytes appeared at a time of a delayed loss of neurons and deterioration in neurological function (Giulian and Robertson, 1990).
4. Suppression of central nervous system inflammation both improved motor neuron survival and preserved motor function (Giulian and Robertson, 1990).
5. Isolation of specific cell populations confirmed that reactive mononuclear phagocytes were the principal source of neuron-killing factors found in the damaged central nervous system (Giulian et al., 1993b).

Reactive microglia appear in almost every type of central nervous system disorder, including infection, trauma, stroke, degeneration, and demyelination. These inflammatory cells are the major source of central nervous system-derived cytokines, help to regulate wound healing in neural tissues, and serve as an important link between systemic immune responses and the central nervous system. Moreover, microglia produce a wide spectrum of cytotoxic agents, some of which demonstrate potent neurotoxic effects. I suggest that inflammatory cell production of neuron-killing factors is a significant pathogenic mechanism common to a variety of central nervous system insults. Inhibition of inflammatory cell-derived toxins may help to preserve neurons near sites of tissue injury and, thus, improve recovery of function.

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45 Antigen presentation by central nervous system glia

HARTMUT WEKERLE

Skin tissue transplanted to the skin of an unrelated recipient is unavoidably rejected within a predictable time of about 7 to 10 days. The same transplant grafted onto the *brain* surface, however, may well survive for unlimited times (Barker and Billingham, 1977). Over many years, this led to the conclusion that the central nervous system is exempt from the mechanisms of immune surveillance that are responsible for identifying and reacting against foreign structures that have invaded the organism.

Several anatomical peculiarities are responsible for the immunoprivileged status of the central nervous system. These include lack of lymphatic drainage, a dense endothelial barrier secluding the brain tissue from blood circulation, and the lack of antigen-presenting cells within the brain (Wekerle et al., 1986).

Yet the brain cannot be completely deprived of immune protection. As in other tissues, there must be ways to control central nervous system infection and neoplastic transformation. Indeed, these pathological lesions are commonly associated with marked infiltrations of inflammatory cells. How do these cells enter the central nervous system, and how do they function there?

Answers to these questions have come from studies of experimental autoimmune reactions involving autoimmune T lymphocytes attacking myelin structures of the brain. This work indicates that the central nervous system *is* controlled and protected by the immune system. Some, though not all, immune cells can pass into the central nervous system, and a considerable number of glial cells can be induced to act as antigen-presenting cells.

EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS: A MODEL FOR CENTRAL NERVOUS SYSTEM IMMUNE REACTIVITY

Experimental autoimmune encephalomyelitis (EAE) is an inducible, myelin-specific autoimmune disease that is commonly considered a valid model for the initial stages of the autoimmune pathogenesis of multiple sclerosis (Lassmann, 1983; Raine, 1984; Wekerle et al., 1986).

EAE lesions are characterized by mononuclear cell infiltrates, which are concentrated around postcapillary microvessels of the central nervous system white matter but also invade the surrounding tissue. These infiltrates are predominantly composed of monocytes and $CD4^+$ T lymphocytes. Typically, the endothelial blood-brain barrier is broken. The results are vasogenic edemas and fibrin deposition. Then local glia cells are highly activated. Astrocytes produce increased amounts of cytoskeletal glial fibrillary acidic protein (GFAP), and microglial cells express major histocompatibility complex (MHC) class I and class II antigens.

In fully developed EAE, the inflammatory lesions are concentrated within the myelinated parts of the central nervous system. It should be noted, however, that, at least in the Lewis rat, the earliest and most severe inflammatory signs are localized within the leptomeninges (Traugott and Raine, 1979; Kitz et al., 1981). In fact, weak EAE may be recognized solely by meningeal infiltration.

Time-course and tissue distribution of EAE are very predictable. Both in actively induced and in passively transferred EAE of the Lewis rats, development of the disease follows a remarkably strict scenario. All models of EAE show a characteristic interval between induction of EAE and the development of clinical manifestations. In actively induced EAE, neurological and histological signs appear 7 to 14 days after immunization. In T line-transferred EAE, the lag phase separating T cell transfer and onset of disease is usually 3 to 4 days (Ben-Nun and Cohen, 1984).

In "classical" EAE of the Lewis rat, the inflammatory process starts in the lumbar spinal cord and then migrates rostrally. The forebrain is attacked only in the most severe cases and in late stages of the disease (Lassmann et al., 1988). The cellular basis of this particular pattern of disease development is uncertain. It has been suggested that in the normal

central nervous system, the number of MHC class II-expressing glial cells is highest in the lumbar spinal cord (Vass and Lassmann, 1990).

T cell-transferred EAE of the Lewis rat, like most other EAE models, is a self-limited monophasic disease (Ben-Nun and Cohen, 1984). Spontaneous remissions are observed not only in normal recipients but even in rats immunosuppressed by heavy irradiation (Sedgwick et al., 1987; Wekerle et al., 1987).

The particular spatial and temporal development of clinical and histological EAE is probably controlled by factors within the central nervous system target tissue. There must be local regulatory mechanisms that define when, where, and how the pathogenic potential of infiltrating T cells is manifested. As discussed below, glial cells interacting with the T lymphocytes appear to be the most probable actors in this interplay.

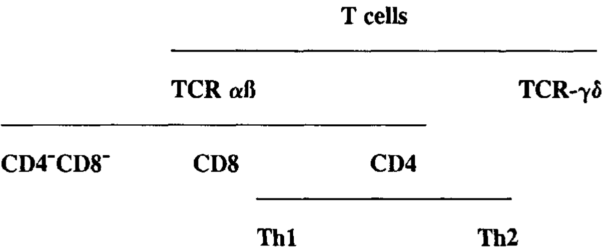

FIG. 45-1. Pedigree of T lymphocytes. The two main classes of T cells are distinguished by their differential usage of T cell receptor genes. The large majority uses the $\alpha\beta$ type, whereas a minor set uses $\gamma\delta$ type of receptors. Among the TCR $\alpha\beta^+$ T cells, cytotoxic T cells express the CD8 membrane marker, while T cells involved in B cell help and delayed type hypersensitivity express the CD4 marker; a third category of unknown physiological function expresses neither CD4 nor CD8. Among the $CD4^+$ T-helper subset, upon activation the Th1 population preferentially secretes the cytokines IL-2 and interferon-γ, whereas, in contrast, Th2 lymphocytes secrete IL-4 and IL-5.

ACTIVATED $CD4^+$ T CELLS CAUSE AUTOIMMUNE EFFECTS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS AND EXPERIMENTAL AUTOIMMUNE NEURITIS

EAE was the first autoimmune disease to be unequivocally transferred by immune cells rather than autoantibodies. This was done first in outbred rats (Koprowski et al., 1960; Paterson, 1960) and subsequently in inbred Lewis rats (Paterson and Bell, 1962).

The T cell nature of the pathogenic, encephalitogenic lymphocytes was revealed by several approaches. First, it was observed that, depleting rats (Gonatas and Howard, 1974) or mice (Bernard et al., 1976) of T lymphocytes rendered them resistant to active induction of EAE using conventional immunization protocols. Reconstitution of T cells restored inducibility of EAE (Ortiz-Ortiz et al., 1976; Stohl and Gonatas, 1980).

More detailed identification of the pathogenic T cells was achieved by combining T cell transfers with monoclonal antibodies binding to T cell subset markers (Fig. 45-1). Transfer of unseparated, *in vivo*-primed lymphocytes, which had been reactivated *in vitro* by confrontation with the encephalitogen myelin basic protein (MBP) showed that the pathogenic potential was associated with T cells carrying markers of the $CD4^+$ subset [high expression of Lyt-1 in the mouse (Pettinelli and McFarlin, 1981) and W3/25 in the rat (Holda and Swanborg, 1982)]. The ultimate proof of the effector function of $CD4^+$ T cells was provided by Ben-Nun et al. (1981) who succeeded in isolating permanent T cell lines that were exclusively specific for MBP. These T cell lines were derived from Lewis rats that had been undergoing EAE after immunization with MBP in Freund's complete adjuvant. They recognized their target epitope in the molecular context of MHC class II antigens, and uniformly expressed the $CD4^+CD8^-$ membrane phenotype (Ben-Nun and Cohen, 1982).

The key role of $CD4^+$ autoaggressive T cells in the pathogenesis of EAE was fully confirmed by therapeutic experiments designed to influence clinical EAE by transferring monoclonal antibodies against different T cell markers. Anti-CD4 antibodies efficiently reduced EAE in rats (Brostoff and Mason, 1984; Brinkman et al., 1985), mice (Waldor et al., 1985), and primates (Rose et al., 1987; Van Lambalgen and Jonker, 1987).

The first successful transfers of EAE used high numbers of lymphocytes freshly isolated from freshly primed immune organs (Paterson, 1960). Soon, however, it became clear that the encephalitogenic potential of MBP-primed T cells could be significantly enhanced by *in vitro* activation with mitogens (Panitch and McFarlin, 1977) and, even more so, by presentation of the specific autoantigen (Richert et al., 1979; Holda et al., 1980).

The definite proof that only activated, but not resting, MBP-specific T cells are able to mediate EAE came again from studies of MBP-specific T cell lines. MBP-specific T cell lines are capable of transferring EAE at extremely low cell numbers, provided they were preactivated *in vitro* by immunospecific presentation of MBP, or, even by polyclonal mitogen. In the *resting* state, however, the same T cell line would

not even induce EAE in 100-fold increased cell numbers (Naparstek et al., 1983).

Activation of autoaggressive T cells is produced in experimental autoimmune disease either *in vivo,* by immunization with strong adjuvants or, in transfer models, by activation *in vitro.* "Spontaneous" autoimmune disease is thought to be the result of T cell activation via reaction of autoimmune T cells with microbial epitopes (Fujinami and Oldstone, 1985). Interestingly, transgenic mice expressing an MBP-specific T cell receptor on all T cells developed "spontaneous" EAE under conventional, but not germ-free, conditions (Goverman et al., 1993). Also T cell activation by (bacterial) superantigens could play a role in parainfectious autoimmunity. Superantigens specific for T cell receptor isotypes common to a particular class of autoimmune T cells could trigger an autoimmune disease by antigen-independent activation (Herman et al., 1991). In fact, this pathway was directly confirmed by recent experiments activating the encephalitogenic potential of MBP-specific T cells by staphylococcal exotoxin (Rott et al., 1992).

Thus, cell activation is critically required to convert the harmless resting autoreactive T cells contained within the normal immune repertoire to pathogenic cells that attack the body's own tissues. Prevention of activation must be one way to ensure self-tolerance.

INTERACTION OF AUTOIMMUNE T CELLS WITH THE ENDOTHELIAL BLOOD-BRAIN BARRIER

In all models of EAE, the pathogenic, myelin-specific T cells must be activated outside the central nervous system, and then have to reach their target tissue via blood. How can these circulating T cells reach the brain tissue that is separated from the circulation by the hermetically closed endothelial blood-brain barrier (BBB)?

The endothelium of the BBB differs from most other endothelia by a number of subcellular specializations (Dermietzel and Krause, 1991) that exclude bloodborne macromolecules and cells from the central nervous system parenchyme. Most important for T cell migration is the network of tight junctions that interconnects adjacent BBB endothelial cells (Brightman and Reese, 1969). These junctions are most elaborated in small postcapillary venules (Nagy et al., 1984).

Until a few years ago, one assumed that the BBB is impermeable for all circulating lymphocytes (Barker and Billingham, 1977). More recently, our own work (Wekerle et al., 1986) and results from others (Hickey et al., 1991) showed that *activated* T line lymphocytes can cross through the BBB, irrespective of their antigen specificity. In contrast, the BBB is indeed completely impermeable to *resting* T cells.

The migration pattern of activated T lymphocytes differs fundamentally from that of resting T cells. Activated T cells, for example, have a propensity to "home" to the thymus (Naparstek et al., 1982) and to perithymic lymph nodes (Klinkert, 1987). In contrast, resting T cells adhere preferentially to high endothelial venules of immune organs, gut or skin (Butcher, 1990).

It seems that distinct profiles of cell adhesion molecules account for the differential migration of activated and resting T cells (Butcher, 1990; Shimizu et al., 1992), although the exact nature of these determinants remains obscure to date.

It should be stressed that the events described so far concern exclusively the *first* contacts between autoimmune T cells and the *naive,* resting BBB endothelium. In the course of the ongoing autoimmune inflammation of the central nervous system, a multitude of cytokines and other inflammatory factors are produced, which activate the local BBB endothelium. The BBB becomes leaky, and plasma macromolecules and blood cells enter to produce edema. The rupture of the BBB, together with chemotactic cytokines, pave the way for cellular infiltrations (Cross et al., 1991).

Identification of the endothelial signals leading to the entry of activated (auto-) immune T cells into the central nervous system will be of high clinical importance. Blocking of these structures would be desirable in treating central nervous system autoimmune disease, whereas their induction or enhancement could have a beneficial effect in infection and neoplasia. Indeed, very recently, monoclonal antibodies against the endothelial $\alpha4\beta1$ integrin were found to interfere with development of EAE in mice (Yednock et al., 1992).

T CELL INTERACTION WITH ASTROGLIA

The tissue-specific autoimmune T cells mediate disease that is quite strictly limited to the relevant target tissue. MBP-specific T cells, for example, attack almost exclusively central nervous system white matter (Raine, 1985), but ignore peripheral nerves. Conversely, T cells recognizing peripheral myelin protein, P2, cause disease only in peripheral nerves (Izumo et al., 1985). Furthermore, T cells recognizing retinal S antigen (Kalsow and Wacker, 1978) or the interphotoreceptor protein (Chan et al., 1987) cause inflammation only in the retina (and in the

pineal gland, which also expresses these two autoantigens).

How can the autoimmune T lymphocytes recognize their target organ so clearly? The most probable explanation should be presentation and recognition of specific autoantigenic epitopes within the tissue. In the case of EAE, this would require the existence of local antigen presenting cells within the central nervous system, a situation clearly negated by the conventional dogma of immune privilege.

It is clear at present that antigen-presenting cells are not only present within the central nervous system, but a considerable proportion of all brain glial cells can function at least partially as competent antigen-presenting cells. Astrocytes were the first glial cells identified as brain cells inducible to MHC products. This was done using brain cultures from neonatal mice. The cells of these cultures did not express any demonstrable class II material. Upon treatment with interferon-γ, however, at least some of the astrocytes became strongly class II-positive (Hirsch et al., 1983; Wong et al., 1984). In addition to MHC products, proinflammatory cytokines induce a variety of cell adhesion molecules on astrocytes. For example, interferon-γ, tumor necrosis factor-α (TNF-α), and interleukin-1 (IL-1) trigger the expression of intercellular adhesion molecule-1 (ICAM-1) (Frohman et al., 1989), lumphocyte function associated antigen-3 (LFA-3), vascular cell adhesion molecule (VCAM), and very late antigen (VLA) (Aloisi et al., 1992; Hurwitz et al., 1992). MHC inducibility on astrocytes by cytokines strongly varies between species and even strains. EAE-susceptible mice and rats often show a higher response to interferon-γ than resistant animals (Massa et al., 1987b), but this association is not complete (Barish and Raissdana, 1990).

MHC class II products and cell adhesion molecules are not only induced in astrocytes by T cell-derived cytokines, but also by certain viruses. Lewis rat astrocyte cultures infected with coronavirus express both high doses of MHC class II antigens (Massa et al., 1986) and ICAM-1 (Kraus et al., 1992). This induction is further enhanced by treatment with TNF-α (Massa et al., 1987a). In the mouse, flaviviruses were reported to induce MHC class II in astrocytes (Liu et al., 1989), and HTLV-I (human T cell leukemia virus) in human astrocytes (Hirayama et al., 1988).

Several agents are known to reduce, or even suppress MHC class II expression on astrocytes. Interferon-β interferes with MHC class II induction on neoplastic (Joseph et al., 1988) and untransformed human astrocytes, presumably acting on transcriptional level (Ransohoff et al., 1991). Transforming growth factor-beta (TGF-β) (discussed in more detail below) is another cytokine reducing astrocytic MHC expression (Schluesener, 1990). Downregulation of MHC in astrocytes has been observed, using β-adrenergic agents like norepinephrine (Frohman et al., 1988b), the neuropeptides vasoactive intestinal peptide (VIP) (Frohman et al., 1988a), glutamate (Lee et al., 1992), potassium channel blockers (Ohira et al., 1991), and, in general, agents that interfere with the function of second messengers (Sasaki et al., 1990). Although the concentrations used in some of these studies seem higher than possibly reached *in situ,* neurotransmitters may have a role in physiological regulation of MHC expression. In experiments culturing cerebellar neurons with astrocytes, glial MHC induction was significantly reduced (Aloisi and Wekerle, 1990).

Expression of MHC products is necessary, but not sufficient for a cell to act as an antigen-presenting cell. Activation of astrocytes by interferon-γ clearly equips the glial cells with the capacity of efficiently presenting protein antigens to specific T lymphocytes (Fig. 45-2). This was first shown in a culture system composed of astrocytes from neonatal Lewis rat brains, and monospecific syngeneic T cell lines (Fontana et al., 1984). In the absence of antigen, the T lymphocytes do not form any particular cellular complexes with the astrocytes. After addition of specific protein (auto-)antigen, however, the T cells start to firmly adhere to the astrocytes, transform to large lymphoblasts and proliferate. This activation reflects recognition of antigen presented by astrocytes, because it depends on the dose of antigen added and on the induction of MHC class II antigens on the astrocytes. The reaction can be completely blocked by monoclonal antibodies binding to the proper MHC class II determinants, but not class I products (Fontana et al., 1984; Fierz et al., 1985). Although astrocytes are good antigen-presenting cells *in vitro,* it should be stressed that they do not possess the *full* functional spectrum of a professional antigen-presenting cell. Astrocytes present antigen optimally only to *primed,* memory T cells, but not to *naive* T cells (Sedgwick et al., 1991a). They seem to lack proper costimulatory factors, such as the B7 molecule required to trigger the full activation program of naive T lymphocytes (Linsley and Ledbetter, 1993).

The interaction between T cells and antigen-presenting astrocytes is by no means unidirectional. Astrocytes act on T cells by presenting antigen. Activated T cells, in turn, affect astrocytes, either via soluble cytokines, or by direct contact. They secrete proinflammatory cytokines (e.g., interferon-γ or TNF-α), which may promote, in addition to the in-

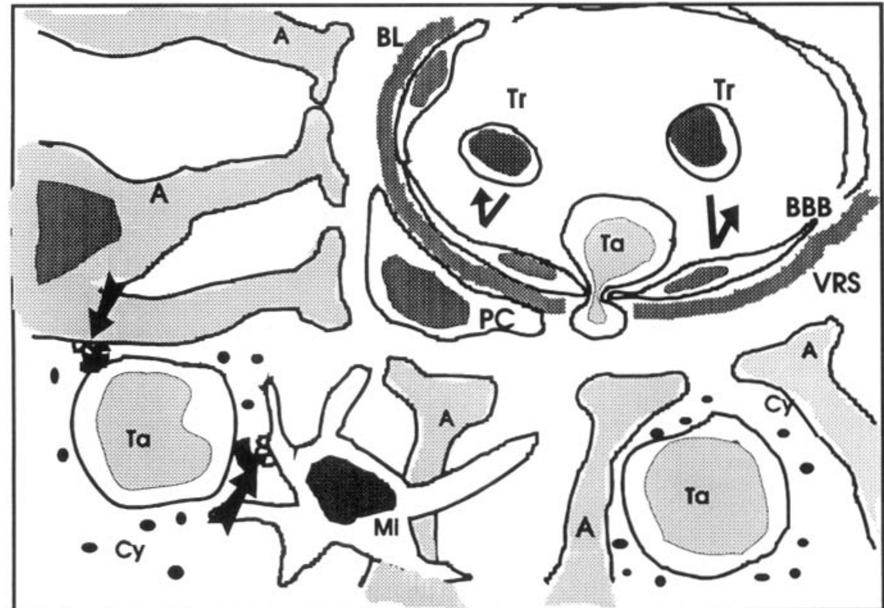

FIG. 45-2. Immunosurveillance of the central nervous system. Activated (Ta), but not resting T cells (Tr) pass through the endothelial blood-brain barrier (BBB) irrespective of their antigen specificity. They break through the microvascular basal lamina (BL), and enter the Virchow-Robin space (VRS) passing by perivascular cells (PC). Some activated T cells penetrate the perivascular glia limitans formed by astrocytic endfeet (A) and microglia (Mi). Proinflammatory cytokines (Cy) released by the migratory activated T cells induce MHC antigens in local glial cells, thus creating the structural basis for antigen presentation to the T cells (arrows).

duction of MHC and cell adhesion molecule expression, glial proliferation, and fibrosis (Selmaj et al., 1991; Yong et al., 1991).

Direct cell-to-cell contact is required for cytotoxic interaction between encephalitogenic T cells and MBP-presenting astrocytes. Studying the coculture system of Lewis rat T cell lines and astrocytes, Sun and his colleagues observed that T cell activation following recognition of the MBP epitope 68-88 resulted in the complete destruction of the antigen-presenting astrocyte monolayers (Sun and Wekerle, 1986). Like T cell activation, target cell lysis depends on the antigen dose (requiring somewhat higher protein concentrations than triggering of proliferation), depends on MHC class II induction on the target cells, and can be completely blocked by relevant monoclonal antibodies. Encephalitogenic T cells not only kill antigen-presenting astrocytes, but all antigen-presenting cells presenting MBP in recognizable molecular context, for example, macrophages, as well. Furthermore, at least in the Lewis rats, cytotoxicity is a property of encephalitogenic T cells. All encephalitogenic T cells have a cytotoxic potential, whereas many nonencephalitogenic T cells are nonlytic (Sun and Wekerle, 1986). Similar observations have been made in murine (Fallis and McFarlin, 1989) and human (Dhib-Jalbut et al., 1990) culture systems, although human $CD4^+$ T cells seem to have a tendency to kill astrocytes even in the absence of nominal antigen (Ruijs et al., 1993).

The previous findings establish that the central nervous system does contain large numbers of autochthonous cells that can be induced to present (auto-)antigens to T cells. Activated MBP-specific T cells that may have crossed the BBB, may interact with local glial cells *in situ* as they have been shown to do *in vitro*. They may induce MHC products and trigger the antigen-presenting potential of astrocytes. Recognition of the appropriate peptide epitope in context of the proper class II product would then result in cytokine production and a cytotoxic attack by T cells against the local autoantigen presenting cells (Sun and Wekerle, 1986).

Although macrophages are presumably required to produce the full pathogenic program of EAE (Brosnan et al., 1981; Huitinga et al., 1990), successful transfer of encephalitogenic T cells into immunosuppressed recipients indicates the autonomous pathogenic potential of the autoaggressive lymphocytes (Sedgwick et al., 1987).

Class II expression on astrocytes *in vivo* is not demonstrable in all cases (Matsumoto et al., 1989), and certainly is lower than on microglial cells (see below). In a careful study combining immunocytochemistry with electron microscopy, Vass and Lassmann (1990) demonstrated interferon-γ-induced class II products on astrocytes. Intriguingly, these molecules were concentrated on the perivascular processes (Vass and Lassmann, 1990). Class II-positive astrocytes have been described in human multiple sclerosis tissues as well (Lee et al., 1990).

CENTRAL ROLE OF MICROGLIA AS ANTIGEN-PRESENTING CELLS

It is now clear, however, that astrocytes are not the only, and perhaps not even the "best," inducible

antigen-presenting cells within the central nervous system. There is good evidence suggesting that microglia cells count among the most efficient antigen-presenting cells of the brain parenchyme. First, Frei and colleagues (1987) showed directly how efficiently microglial cells process and present protein antigens to syngeneic T cells *in vitro*. Resting microglia cells isolated mechanically from mixed glial cultures of newborn mouse brain readily take up particulate material and express receptors for immunoglobulin Fc parts, but no MHC antigens. Interferon-γ treatment leads to the expression of MHC class II products (Frei et al., 1987; Suzumura et al., 1987), and to the capacity of presenting protein antigens to MHC-compatible T cells (Frei et al., 1987). Similar findings were made in rat cultures (Woodroofe et al., 1989).

Microglia are the most prominent MHC-expressing central nervous system cells *in vivo*. Intravenous infusion of recombinant interferon-γ into rats preferentially induces MHC antigens in microglia in some (Steiniger and Van der Meide, 1988), but not in other studies (Momburg et al., 1986). Clearly, however, profound changes in membrane antigen expression on glial cells are caused by direct, intrathecal injection of cytokines. Vass and Lassmann found that infusion of recombinant mouse interferon-γ into the subarachnoidal space of Sprague-Dawley rats resulted in typical patterns of MHC induction. The first cells to be induced were perivascular dendritic cells and parenchymal microglia. At higher doses and at later stages only astrocytes and ependymal cells became MHC-positive (Vass and Lassmann, 1990).

Microglial MHC expression can be triggered by multiple pathways. Immunocytochemical analyses of MHC class II induction in central nervous system parenchymal cells established that microglial cells exhibit the strongest class II expression in autoimmune inflammation (Matsumoto et al., 1986; Konno et al., 1989; Sedgwick et al., 1991b). As discussed above, most of this induction seems to be due to the local release of proinflammatory cytokines from autoimmune infiltrate cells (Kennedy et al., 1992; Merrill et al., 1992).

There are, however, additional noninflammatory pathways to induce MHC antigens in microglial cells. Cell degeneration in general leads to preferential induction of MHC products in central nervous system glial cells, preferentially in microglial cells. In 1966 Kreutzberg demonstrated that dissection of a rat facial nerve is followed by the activation of perineuronal microglial cells positioned within the central nervous system motor nuclei. The microglial reaction was noted only ipsilaterally, but not on the contralateral nucleus, with an intact peripheral nerve. Microglia activation is reflected by proliferation and by the increased expression of complement receptors (Graeber et al., 1988). Most important, however, the activated microglial cells are induced to express MHC class I (Maehlen et al., 1988) and class II determinants (Streit et al., 1989b). Very similar effects have been observed after transection of sciatic nerves (Maehlen et al., 1988; Gehrmann et al., 1991).

The molecular basis for microglial activation and MHC induction has remained uncertain. Immunocytochemistry suggested that the lesioned neurons start to produce mediators related to interferon-γ (Olsson et al., 1989). This could not be confirmed in a more detailed subsequent study. Western and Northern blot analyses failed to demonstrate interferon-γ (Kiefer et al., 1991).

Remote activation of central nervous system microglia has been achieved by peripheral axotomy, but it also is seen by neuronal poisoning following retrograde transport of ricin (Streit et al., 1989a), and ischemia (Gehrmann et al., 1992a). Perhaps most surprisingly, microglial cells in the *central nervous system* are activated during autoimmune attacks against *peripheral* nerves. Gehrmann and coworkers induced experimental autoimmune neuritis in the Lewis rat by transferring neuritogenic T cell lines recognizing peripheral myelin protein, P2 (Gehrmann et al., 1992b). As mentioned, inflammation in this disease is strictly confined to the peripheral nervous system, sparing completely the central nervous system (Izumo et al., 1985), and follows an equally rigid time schedule. Infiltration and neurological deficits start on the fourth day after cell transfer (Heininger et al., 1986). Two days *before* development of experimental autoimmune neuritis, microglial cells around central nervous system motor neurons become activated, expressing MHC antigens. The activation gradually intensifies and spreads rostrally, finally to reach nuclei gracilis and cuneatus (Gehrmann et al., 1992b).

OTHER ANTIGEN-PRESENTING CELLS IN THE CENTRAL NERVOUS SYSTEM?

Among all central nervous system cell lineages, neurons are least inducible to induction of MHC antigens. Neurons are resistant to activation by interferon-γ in culture, under conditions, where all other cell types express at least class I determinants (Wong et al., 1984). In fact, this lack of MHC expression seems to be responsible for persistence of neurotropic viruses within the central nervous system. Neurons harboring lymphocytic choriomeningitis virus are resistant

against the cytotoxic effects of virus-specific $CD8^+$ killer T cells. They are efficiently lysed, however, after transfection with suitable MHC class I determinants (Joly et al., 1991).

The immunological status of oligodendrocytes is more complex. *In vitro,* MHC class I, but not class II antigens are inducible on rodent oligodendrocytes by interferon-γ. In most of these studies, membrane markers like galactocerebroside (GalC) were used as markers of cell maturity (Lisak et al., 1984; Wong et al., 1984). Induction of MHC class I is accompanied by the appearance of cell adhesion molecules (Satoh et al., 1991). Also within multiple sclerosis plaques, residual oligodendrocytes failed to bind class II-specific antibodies (Lee and Raine, 1989).

Interferon-γ inducibility of class II seems to be lost during rodent oligodendrocyte differentiation. In contrast to mature oligodendrocytes, bipotential O-2A glia progenitor cells are still inducible in culture (Calder et al., 1988). According to a recent report, class II induction can be restored in GalC- and proteolipid protein-positive rat oligodendrocyte cultures by glucocorticoids (Bergsteinsdottir et al., 1992).

At present, it appears that neurons are incapable of presenting antigen to any T lymphocyte class. In contrast, oligodendrocytes fulfil the criteria for presenting peptides in MHC class I context, and thus to interact with $CD8^+$ cytotoxic T cells. Whether they are able to interact with $CD4^+$ T cells under regular conditions remains to be proven.

ANTIGEN PRESENTATION *IN VIVO*

Are the class II-induced glial cells indeed involved in the *in vivo* presentation of (auto-)antigens to specific T lymphocytes? Maehlen et al. (1989) showed that this is probably the case. Inducing EAE in facial nerve axotomized Lewis rats by active immunization with MBP in complete Freund's adjuvant they found mononuclear infiltration in the lesioned facial nucleus, but not in the normal, contralateral area (Maehlen et al., 1989). Others extended these findings using optic tract lesions and passive T cell transfer EAE (Konno et al., 1990).

Less clear is the situation with cytokine-activated glia. Billiau and colleagues (1988) were the first to show that systemic treatment of SJL/J mice with interferon-γ did by no means enhance active induction of EAE, but rather decreased development of disease. Monoclonal antibodies against interferon-γ also enhanced chronic relapsing EAE in SJL/J mice after active or passive induction (Duong et al., 1992). A similar paradoxical effect of interferon-γ (and of antiinterferon antibodies) was noted in Lewis rats after intraventricular cytokine instillation, although in the same studies intravenous treatment preceding the onset of disease aggravated EAE (Voorthuis et al., 1990). These observations are not easy to explain. Observations in related autoimmune models emphasize the diversity of effects potentially exerted by interferon-γ (Hartung et al., 1990). Considering the pleiotropic effects of interferon-γ, many factors affecting EAE development may be altered: T cell activation outside the central nervous system, migration through the BBB and, finally, interaction with parenchymal target cells. Some of these effects may neutralize each other. Thus, timing and dosaging of cytokine treatment crucially determines the outcome of the treatment. However, most importantly, treatment of patients with interferon-γ provoked a rate of relapses, which cogently demonstrated the proinflammatory effect of the cytokine in a human central nervous system disease with putative autoimmune pathogenesis (Panitch et al., 1987).

Activation of glial cells by degenerative events which may occur within the central nervous system, or even outside, is certainly of importance to understand human disease. That similar events do take place in human brains has been demonstrated under various pathological conditions. In multiple sclerosis lesions, for example, MHC class II induction extends into non-infiltrated "healthy" parenchyme areas surrounding actual lesions (Woodroofe et al., 1986; Boyle and McGeer, 1990). Further, MHC-positive glial cells are characteristic for degenerative disorders as diverse as Huntington's chorea, Parkinson's and Alzheimer's disease (McGeer et al., 1988; Sobel and Ames, 1988; Styren et al., 1990; Perlmutter et al., 1992).

WHICH ARE THE MOST IMPORTANT NEURAL ANTIGEN-PRESENTING CELLS?

As pointed out above, Vass and Lassmann demonstrated a hierarchic sequence of glial class II induction following intrathecal treatment with interferon-γ. They found that in a first stage of the response, perivascular cells and microglia are induced, and that only later astrocytes and ependymal cells follow with MHC class II expression (Vass and Lassmann, 1990).

Intriguing information on the role of antigen-presenting cells in generation of EAE came from studies using bone-marrow reconstituted irradiation chimeras. Studies of chimeric rats established that most *parenchymal* microglial cells were of recipient

origin. In MHC disparate rat chimeras, bone-marrow derived MHC class I determinants were not found within the parenchyme (Matsumoto and Fujiwara, 1987), and similar findings were made using mouse chimeras with a transgenic bone-marrow marker (De Groot et al., 1992). These observations leave it open, whether they are due to the neuroectodermal origin of most parenchymal microglial cells, or to their extremely slow turn over.

On the other hand, similar studies led to the identification of a new, bone-marrow derived perivascular glia population. Perivascular microglial cells extend processes between astrocytic endfeet to form local glia limitans. They are highly susceptible to bloodborne interferon-γ (Lassmann et al., 1991). Interestingly the same cells are activated in areas close to neuronal degeneration (Streit et al., 1989a). Their active role in antigen presentation *in situ* has been established in transfers of parental MBP specific T cells to rat chimeras. MHC compatibility of the transferred encephalitogenic T cells with the chimeras' bone marrow was necessary and sufficient to produce clinical and histological EAE (Hinrichs et al., 1987; Hickey and Kimura, 1988; Matsumoto and Fujiwara, 1988). Since, obviously, astrocytes and most resident microglial cells would lack suitable MHC class II products to present myelin to the transferred T cells, the bone marrow-derived perivascular glial cells remain as the most probable candidate antigen-presenting cells to produce EAE in these animals.

Together the data suggest that, in principle, several intracerebral antigen-presenting cells may have an active role in the development of EAE. While resting endothelial cells do not seem to provide antigen-specific signals to circulating activated T cells, antigen may be presented by perivascular glial cells. Their high sensitivity to MHC inductive stimuli and their strategic location close to the BBB predestine these bone marrow-derived glial cells to control the very first steps in immunosurveillance and autoimmunity of the central nervous system parenchyme. When inflammation develops, additional antigen-presenting cells may be recruited, including bone marrow-independent parenchymal microglia, and finally astrocytes. At present it is uncertain, whether oligodendrocytes can also be induced to act as antigen-presenting cells *in vivo*. Antigen presentation by astrocytes may well occur only in late stages of the autoimmune inflammatory process. In view of the crucial physiological roles of astrocytes, cytotoxic effects by encephalitogenic T cells against these cells would be especially disastrous, and could result in severe clinical deficits.

SUPPRESSOR ASTROCYTES-T CELL APOPTOSIS

Not all astrocytes are fully efficient antigen-presenting cells. As discussed above, the genetically determined variation in astrocytic MHC class II inducibility is considerable. However, even astrocytes from animals with high MHC inducibility vary in their antigen presenting capacity. Screening a series of Lewis rat-derived cloned astrocyte lines, we found that many of these lines triggered antigen-specific T cell proliferation. Other astrocyte clones, however, did not present productively protein antigens, although they expressed cytokine-induced MHC class II determinants. In most cases this was not due to "simple" inability to process the proteins, but reflected active suppression of T cell proliferation. These suppressive astrocytes vigorously suppressed even T cell responses elicited by "professional" antigen-presenting cells (dendritic cells) derived from peripheral immune organs (Wekerle et al., 1987). Similar cell dose-dependent suppression had been recorded in a related rat (Matsumoto et al., 1992), and mouse systems (Takiguchi and Frelinger, 1986), and more recently with human astrocytes and T cells (E. Meinl, personal communication).

Suppressive control of T cell activation is not a function limited to cerebral astrocytes, but similar activities were described in antigen presentation within the eye. Retinal Müller cells (Caspi et al., 1987), and ciliary body cells (Helbig et al., 1990) also have been shown to suppress antigen presentation to T cells.

The molecular mechanism of astroglial suppression of T cell activation remain to be elucidated. Astrocytes may act on T cells either directly by cell-to-cell contacts, or via suppressive mediators. In principle, any astrocyte product with known immunosuppressive activity could be involved in the process.

Early studies indicated apolipoprotein E (ApoE) as a possible immunosuppressive agent of astrocytes. ApoE, a plasma protein involved in lipid transport and metabolism, suppresses cellular immune responses, though at relatively high concentrations (Mahley, 1988). ApoE is constitutively expressed in astrocytes *in vivo* (Boyles et al., 1985), and *in vitro* (Oropeza et al., 1987). The molecule is involved in nerve de- and regeneration (Ignatius et al., 1986; Snipes et al., 1986). In astrocyte cultures its expression is further enhanced by interferon-γ treatment (Oropeza et al., 1987).

More recently, members of the transforming growth factor-β (TGF-β) family have attracted attention as possible downregulators of central nervous system immune reactivity. Glial TGF-β was discovered in analyses of a soluble immunosuppressive

mediator released by transformed human glioblastoma cells (Schwyzer and Fontana, 1985). Its molecular identity as TGF-β2 was revealed by protein sequencing and by cloning of its gene (De Martin et al., 1987). While original studies suggested that TGF-β2 is exclusively released from glioma tissue, later at least the latent form of TGF-β2 was demonstrated in cultures of normal astrocytes (Saad et al., 1991; Constam et al., 1992). TGF-β, whose production seems to involve autocrine regulation (Saad et al., 1991), has profound effects on central nervous system differentiation and function. For example, TGF-β1 enhances nerve growth factor production (Lindholm et al., 1990), and cell adhesion molecule expression (Saad et al., 1991) in rat astrocytes. It is induced in astrocytes by trauma (Lindholm et al., 1992).

Most important in our context, TGF-β2, like other isoforms of TGF-β, is a powerful immunosuppressive agent (Schwyzer and Fontana, 1985; Schluesener and Lider, 1989) that acts on several levels. TGF-β reduces MHC class inducibility of glial cells (Schluesener, 1990), and suppresses T cell activation and proliferation (Siepl et al., 1988; Wahl et al., 1988). Interestingly, TGF-β may act as a mediator of post-EAE suppressor T cells (Karpus and Swanborg, 1991), and may downregulate autoimmune T cell responses after oral tolerization (Kuruvilla et al., 1991; Khoury et al., 1992). Finally, TGF-β expression is enhanced in the spontaneous recovery phase of EAE, further emphasizing its potential role as a downregulatory agent (Khoury et al., 1992).

TGF-β thus looks like an ideal drug to treat T cell-mediated autoimmune diseases. Indeed there are numerous reports on successful treatment of EAE (Johns et al., 1991; Kuruvilla et al., 1991; Racke et al., 1991) and other central nervous system inflammations (Stitz et al., 1991).

Nevertheless, TGF-β is by no means a completely reliable antiinflammatory drug, and this is due to several undesirable properties. TGF-β displays marked and broad chemotactic activity. It attracts monocytes (Wahl et al., 1987), polymorphonuclear leukocytes (Reibman et al., 1991), and T lymphocytes (Adams et al., 1991). It may hence not surprise that under certain conditions, treatment with TGF-β may not result in suppression of an (autoimmune) inflammation, but rather in its exacerbation (Drake and Issekutz, 1993). Furthermore, TGF-β induces preferential immunoglobulin isotype production by B lymphocytes (in the mouse IgA (Coffman et al., 1989), and IgG2b (McIntyre et al., 1993)), which may be deleterious under specific conditions.

Most recently, IL-10 has raised attention as a potential downregulatory cytokine of the central nervous system. Predominant among the many effects of IL-10 are efficient inhibition of cytokine production by monocytes (De Waal Malefijt et al., 1991), and functional suppression of T cells, especially the Th1 subset (Del Prete et al., 1993), which is involved in EAE mediation. Like TGF-β, IL-10 expression comes up in the late, recovery stages of SJL/J mouse EAE (Kennedy et al., 1992). This and the recent demonstration of IL-10 in human astrocyte cultures (E. Meinl, personal communication), make this cytokine an additional possible mediator of astrocyte-dependent immunosuppression.

Recent work raised the possibility that many of the activated T cells entering the central nervous system may not leave that tissue, but rather will perish there. Ohmori et al. (1992), for example, found that T cells having entered the central nervous system do not persist for a long time in their state of activation and proliferation. Moreover, a morphological study of EAE lesions in rat brains indicated that numerous round cells undergo apoptosis. Pender et al. (1991) concluded that most of these cells were of oligodendrocytic origin. Combining immunocytochemistry, electron microscopy, and *in situ* nick translation of apoptotic DNA (Gold et al., 1993), Lassmann and colleagues identified most of the apoptotic cells in EAE as degenerating T cells (Schmied et al., 1993). These findings might explain the remarkably low frequency of autoantigen specific T cells recoverable from EAE brains. Furthermore, similar patterns of cell death have been observed in active infiltrations of multiple sclerosis brain lesions (H. Lassmann, personal communication). These findings certainly have to be considered in interpreting T cell receptor sequences amplified by polymerase chain reaction from multiple sclerosis brain tissue (Oksenberg et al., 1993). Apoptosis of activated, myelin specific T cells may lead to a profound distortion of the T cell repertoire in brain infiltrates, with a relative underrepresentation of the pathogenic T cells among unspecific infiltrate lymphocytes. Further, it will remain to be established, whether related processes are involved in "determinant spreading" described by Lehmann et al. (1992).

There is evidence that T cell suppression by astrocytes is not just a tissue culture artifact, but may have a "real" function *in vivo*. Equivalent mechanisms may be responsible for the unexpectedly low proliferation rate noted for intraparenchymal infiltrating T cells in transfer EAE (Ohmori et al., 1992).

The central nervous system parenchyme is a microenvironment, which can be induced to interact positively with transmigrating T lymphocytes. Glial cells, especially microglia, but also certain astrocytes can be induced to present protein (auto-)antigens in

MHC class II context to specific T cells. T cell activation is under tight control, with regulatory mechanisms making sure that it is limited both in duration and in intensity.

CONCLUSION

All experimental evidence indicates that the central nervous system is subject to active immune surveillance. The surveillant immune cells are activated, that is, they must have been recently primed by encountering "their" antigen. Within the central nervous system parenchyme, several glial cell types are able to present protein antigen to T cells. Microglial cells appear to be the most promptly inducible and efficient central nervous system antigen-presenting cells. In addition, at least some astrocytes can be recruited to positively interact with T cells. Many astrocytes seem, however, to be involved in downregulation of immune responses. They produce mediators that suppress topical MHC induction and reduce activity of T cells. Further, astrocytes are capable of causing apoptosis, preferentially in activated T cells that interact with locally presented (auto-)antigen.

The complex and tight control of immune reactivity within the central nervous system may well have developed to maximally reduce bystander damage to the sensitive neural cells.

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46 | Cytokine production

ETTY N. BENVENISTE

This chapter emphasizes the expression and action of a select group of cytokines, namely, those implicated in contributing to inflammatory and immune responses within the central nervous system. These cytokines include interferon-gamma (IFN-γ), interleukin-1 (IL-1), tumor necrosis factor alpha and beta (TNF-α/TNF-β), interleukin-6 (IL-6), transforming growth factor beta (TGF-β), the colony stimulating factors (CsFs), and the chemoattractant cytokines called chemokines. The chapter's main focus is on how glial cells both respond to and synthesize cytokines, and how these changes in gene expression and function may contribute to various neurologic disease states such as multiple sclerosis, experimental allergic encephalomyelitis (EAE), and AIDS dementia complex.

BACKGROUND

The central nervous system is generally regarded as an "immunologically privileged site" for two reasons: (1) the central nervous system is for the most part devoid of a lymphatic system that captures potential antigens, and (2) the central nervous system is protected from circulating blood by the blood-brain barrier, a specialized vasculature consisting of endothelial cells with tight junctions, which is impermeable to immunoglobulins and cytokines and which acts to restrict the migration of lymphoid cells into the central nervous system. Additionally, cells of the central nervous system (neurons, astrocytes, oligodendrocytes, microglia) constitutively express very low levels of class I and II major histocompatibility complex (MHC) antigens, which play a fundamental role in the induction and regulation of immune responses (Wong et al., 1984a. See Chapter 45). Pathological events within the central nervous system often result in a breakdown of the blood-brain barrier, which permits cells of the peripheral immune system access to this site. In human diseases such as multiple sclerosis (Hauser et al., 1983) and AIDS dementia complex (Navia et al., 1986) and in animal models of central nervous system disease such as EAE (Raine, 1984), inflammatory infiltrates composed of varying ratios of activated T cells, B cells, and macrophages are found in the brain.

Studies have addressed the question whether factors from immune cells contribute to astrogliosis. Astrogliosis is the result of astrocyte proliferation, hypertrophy, and the increased synthesis of glial fibrillary acidic protein (GFAP), an astrocyte-specific protein that eventually produces dense glial scars in the central nervous system. Astrogliosis is often associated with inflammatory infiltrates in the central nervous system and is one of the characteristic hallmarks of the diseases multiple sclerosis and AIDS dementia complex. Because of the close proximity of astrocytes and activated lymphocytes in the central nervous system, the question arose as to whether lymphocytes might contribute to the process of astrogliosis. To test this hypothesis, Fontana et al. (1982a, 1982c) examined the ability of lymphocytes to produce soluble products that might stimulate astrocyte proliferation. Supernatants from activated rat lymphocytes were shown to enhance both RNA and DNA synthesis in rat astrocyte cultures (Fontana et al., 1982a). Merrill et al. (1984) demonstrated that both rat astrocytes and oligodendrocytes could respond by increased proliferation to supernatants from activated human T cells, and these supernatants also enhanced maturation of oligodendrocytes as assessed by the increased expression of myelin basic protein (MBP) (Benveniste et al., 1985).

Since soluble factors from activated lymphoid cells could enhance the growth of glial cells, investigators were interested in determining whether the reciprocal could occur, that is, whether glial cells might secrete soluble products that would affect lymphoid cells and/or macrophages. Fontana et al. (1982b) discovered that cultured murine astrocytes, upon stimulation with lipopolysaccharide, secreted significant amounts of prostaglandin and an IL-1 like factor, and that human glioblastoma cell lines constitutively secreted an IL-1 like molecule. These early studies provided evidence that glial cells and lymphocytes could communicate via soluble mediators.

CYTOKINES: GENERAL FEATURES

Cytokines play a major role in the initiation, propagation, regulation, and suppression of immune and inflammatory responses. Although cytokines comprise a diverse group of proteins, they share a number of general properties. Cytokines are low molecular weight proteins produced during the effector phases of immunity. Most cells do not constitutively produce cytokines; instead, an activation event results in cytokine gene transcription. Cytokines generally are secreted, but can also be expressed on the cell surface. An individual cytokine can be produced by many different cell types and have multiple biological effects on different cell types. Cytokines have also been shown to have redundant functions, that is, several cytokines can mediate a common event. Thus, the cytokine system displays pleiotropism and redundancy. Cytokines often influence both the synthesis and function of other cytokines, resulting in complex "cytokine cascades." Cytokines act locally and initiate their action by binding to specific cell surface receptors on target cells. These receptors show high affinities for their ligands, with dissociation constants in the range of 10^{-10} to 10^{-12} M, suggesting that very low amounts of a cytokine can elicit a biological response.

INTERFERON-GAMMA AND GLIAL CELLS

Interferon-gamma (IFN-γ), a pleiotropic cytokine with antiviral activity, antiproliferative effects, and immunomodulatory effects, is produced predominantly by activated T cells (for review see Farrar and Schreiber, 1993). These immune effects include the ability to enhance the functional activity of macrophages, promotion of T- and B-cell differentiation, and the modulation of both class I and class II major histocompatibility complex (MHC) expression on a wide variety of cells.

Biological Action on Glial Cells

IFN-γ is present in the central nervous system only in disease states in which the blood-brain barrier has been disrupted, and activated T-cells have infiltrated into that site. IFN-γ is a potent modulator of MHC antigen expression in a number of cell types, including astrocytes, oligodendrocytes, and microglia. The preceding chapter provides a comprehensive overview of MHC antigen expression on the astrocyte, so the effect of IFN-γ on glial cell MHC expression will be just briefly discussed here.

Cells in the brain normally express extremely low levels of class I MHC antigens, which have a critical role in the regulation of immune responses. IFN-γ induces an increase in the expression of class I MHC antigens on astrocytes, oligodendrocytes, and microglia both *in vitro* and *in vivo* (Wong et al., 1984a, 1984b; Suzumura et al., 1986). The implication of enhanced class I MHC expression on these cells is that they can be rendered susceptible to lysis by class I-restricted cytotoxic T lymphocytes (Skias et al., 1987).

Class II MHC molecules have a key role in regulating the immune response by presenting antigen to T-helper cells, resulting in their activation and differentiation (for review see Benoist and Mathis, 1990). Astrocytes and microglia do not constitutively express class II MHC antigens; however, IFN-γ can induce class II molecules on these cells *in vitro* (Wong et al., 1984a; Fierz et al., 1985; Suzumura et al., 1987). *In vitro,* class II MHC-positive astrocytes and microglia can present antigen to T cells in an MHC-restricted manner, resulting in T-cell activation (Fierz et al., 1985; Frei et al., 1987). The implication of class II expression on both astrocytes and microglia is that these cells may stimulate the development of aberrant immune responses within the central nervous system.

Recent studies from our laboratory have focused on understanding the intracellular and molecular events involved in IFN-γ induction of class II MHC expression by primary rat astrocytes. Our results indicate that both IFN-γ-induced protein kinase C activity and enhanced sodium influx (via the Na^+/H^+ antiporter) are required for class II MHC gene expression in the astrocyte (Benveniste et al., 1991). Additionally, IFN-γ-induced tyrosine kinase activity is also necessary for class II MHC expression (Lee et al., 1995). The cloning and sequencing of class II MHC genes have led to the identification of conserved sequences located between 50 and 160 base pairs upstream of the transcriptional start site of all class II promoters. Three elements have been described: from 3′ to 5′, they are the Y, X, and W boxes, respectively (for review see Benoist and Mathis, 1990). These conserved elements are necessary for constitutive and IFN-γ-mediated expression of class II MHC in various cell lines. Studies on primary rat astrocytes indicate that IFN-γ acts at the transcriptional level to induce class II expression in these cells, and that the W, X, and Y elements are all essential for IFN-γ inducibility of the class II gene. We have identified a IFN-γ-induced DNA binding protein with specificity for the X element that is produced by astrocytes, named IFNEX (Moses et al., 1992). We believe IFNEX is involved

in IFN-γ induced transcription of the class II MHC gene.

Role in Neurologic Diseases

Due to its ability to modulate expression of MHC antigens on glial cells, and activate macrophages, IFN-γ has been proposed to contribute to the pathogenesis of multiple sclerosis. In clinical trials, administration of IFN-γ to relapsing-remitting multiple sclerosis patients caused exacerbation of disease (Panitch et al., 1987), suggesting that IFN-γ has a role in disease progression. In multiple sclerosis patients, IFN-γ positive cells have been localized to the plaque region, and these cells have been identified as astrocytes (Hofman et al., 1991).

The best characterized experimental model for central nervous system autoimmune disease is EAE. This disease is induced by injection of spinal cord components such as MBP or proteolipid protein (PLP) with adjuvant, injection of MBP or PLP peptides, or transfer of encephalitogenic MBP or PLP-specific T cells to naive recipients. EAE is characterized by an inflammatory infiltration of the central nervous system by activated T cells and macrophages, demyelination, and acute, chronic, or chronic-relapsing paralysis. The mediators of this disease are MBP or PLP-reactive $CD4^+$ T helper cells that are class II MHC-restricted (for review see Zamvil and Steinman, 1990). Interestingly, in mice with EAE, treatment with neutralizing antibody against IFN-γ caused an increase in disease severity and mortality (Billiau et al., 1988; Duong et al., 1992), while treatment with IFN-γ itself resulted in reduced morbidity and mortality (Billiau et al., 1988). These results suggest that IFN-γ exerts an inhibitory effect on the development of EAE, which is different than that which was observed in multiple sclerosis clinical trials, in which IFN-γ led to an exacerbation of disease (Panitch et al., 1987). IFN-γ is found in the central nervous system of mice with EAE (Kennedy et al., 1992; Merrill et al., 1992) and persists into the early chronic/remission stage (Kennedy et al., 1992). The reasons for the contradictory actions of IFN-γ in EAE compared to multiple sclerosis are unknown at this time.

INTERLEUKIN-1 AND GLIAL CELLS

Interleukin-1 (IL-1) is a 17,000 Dalton cytokine produced predominantly by activated macrophages, although other cell types, such as endothelial cells, B cells, keratinocytes, microglia, and astrocytes, can also secrete IL-1 upon stimulation (for review see Arai et al., 1990). IL-1 is responsible for mediating a variety of processes in the host response to microbial and inflammatory diseases. IL-1 is expressed in two major forms, IL-1α and IL-1β, which are the products of two different genes. Although these two forms of IL-1 have less than 30% homology between their amino acid sequences, they both bind to identical receptors and have similar biologic activities. IL-1 is the major costimulator for T-helper cell activation via the augmentation of both IL-2 and IL-2 receptor expression. IL-1 is a principal participant in inflammatory reactions through its induction of other inflammatory metabolites, such as prostaglandin, collagenase, and phospholipase A2. IL-1 acts on endothelial cells to promote leukocyte adhesion and induces the production of various cytokines, such as IL-6, tumor necrosis factor-alpha, colony-stimulating factors, and IL-1 itself.

Biological Action on Glial Cells

Purified IL-1 was shown to have a mitogenic effect on astrocyte growth *in vitro* (Giulian and Lachman, 1985), while IL-1 directly injected into the brain can stimulate astrogliosis (Giulian et al., 1988). These results suggest that IL-1 may contribute to astroglial scarring. Both activated astrocytes and microglia have been shown to secrete IL-1 *in vitro* (see below); thus these cells would provide an endogenous brain source of IL-1.

A variety of central nervous system cells have been shown to produce cytokines in response to IL-1. IL-1 stimulation of primary rat astrocytes primes them for the secretion of tumor necrosis factor-alpha (TNF-α) (Chung and Benveniste, 1990), and induces IL-6 (Frei et al., 1989; Benveniste et al., 1990; Norris et al., 1994) and transforming growth factor-beta (TGF-β) production (da Cunha and Vitkovic, 1992), and primary human astrocytes produce IL-6 and colony-stimulating factors in response to IL-1 (Aloisi et al., 1992; Lee et al., 1993). With respect to microglia and oligodendrocytes, IL-1 induces TGF-β expression by these cells (da Cunha et al., 1993). These are all cytokines involved in mediating immune reactions and inflammatory responses; thus their production by resident brain cells can contribute to these processes within the central nervous system.

Studies from our laboratory have been directed toward understanding the molecular and intracellular signaling events involved in TNF-α gene expression. IL-1 induced TNF-α gene expression in astroglioma cells is dependent on protein kinase C (PKC) activation in that two PKC inhibitors, H7 and staurosporine, abrogate IL-1-induced TNF-α expression,

and depletion of PKC activity by prolonged treatment with a high concentration of phorbol ester (phorbol myristate acetate) renders the astroglioma cells incapable of producing TNF-α in response to IL-1 (Bethea et al., 1992b). We have determined that PKC activity is also required for IFN-γ/IL-1β induction of TNF-α gene expression in rat astrocytes, and that PKC activity is needed for transcriptional activation of the TNF-α gene (Chung et al., 1992).

Expression by Glial Cells

IL-1 production by glial cells of the central nervous system was originally suggested by a study in which cultured murine astrocytes, upon stimulation with lipopolysaccharide (LPS), secreted an IL-1-like factor (Fontana et al., 1982b). This finding was later confirmed using astrocyte cultures of >95% purity, and observing that LPS-stimulated murine astrocytes expressed mRNA for both IL-1α and IL-1β (Malipiero et al., 1990). Additionally, primary cultures of human fetal astrocytes produce IL-1 upon stimulation with LPS (Velasco et al., 1991). Both rat and murine microglia produce IL-1 in response to LPS stimulation (Giulian et al., 1986; Malipiero et al., 1990; Chao et al., 1992). There has been some controversy as to whether primary cultures of astrocytes do in fact produce IL-1, the concern being that contaminating microglia in these cultures may be the source of IL-1. The use of double labeling immunohistochemistry to positively identify cells *in vivo* expressing IL-1 has demonstrated that both astrocytes and microglia express IL-1, but that astrocytes are the more frequent producer of this cytokine in diseased brain (da Cunha et al., 1993). Oligodendrocytes also are capable of producing IL-1 in that human oligodendroglioma cell lines produce IL-1 (Merrill and Matsushima, 1988), and oligodendrocytes in diseased brain stain positively for IL-1 (da Cunha et al., 1993). These data indicate that there are three endogenous sources of IL-1 within the central nervous system: astrocytes, microglia, and oligodendrocytes.

Role in Neurological Diseases

Because of the known inflammatory effects of IL-1 and the fact that glial cells can produce IL-1 within the central nervous system, there has been interest in the role of IL-1 in EAE. Jacobs et al. (1991) demonstrated that *in vivo* administration of IL-1α enhanced the severity and chronicity of clinical paralysis associated with EAE, whereas treatment of animals with soluble mouse IL-1 receptor (an IL-1 antagonist) significantly delayed the onset of EAE, reduced the severity of paralysis, and reduced the duration of disease. A study by Kennedy et al. (1992), examining EAE induced in SJL/J mice by adoptive transfer of MBP-sensitized cells, demonstrated that mRNA for IL-1 was detected in the acute phase of disease and remained elevated during the early chronic state. mRNA for other cytokines such as IL-2, IL-4, IL-6, IL-10, and IFN-γ were also detected in this model of EAE. In a separate study, IL-1 protein expression was detected in the central nervous system of Lewis rats 10 days after immunization with spinal cord homogenate. The number of lesions with IL-1-positive cells increased during full-blown EAE, and declined during remission. Based on microscopic analysis, IL-1 immunoreactivity was detected in macrophages and microglia (Bauer et al., 1993). In contrast, Merrill et al. (1992) did not detect IL-1 in the brains of SJL/J and B10.PL mice that were immunized with MBP peptides. This would suggest that the method of immunization by which EAE is induced may influence cytokine expression in the brain.

TUMOR NECROSIS FACTORS ALPHA AND BETA AND GLIAL CELLS

TNF-α, a 17,000 Dalton peptide, is the principal mediator of the host response to gram-negative bacteria (for review see Vilcek and Lee, 1991). Activated macrophages are the major cellular source for TNF-α, although other cell types such as T cells, mast cells, microglia, and astrocytes can be stimulated to secrete TNF-α. TNF-α is an active participant in inflammatory responses and can alter vascular endothelial cell function by enhancing the permeability and adhesive properties of these cells. TNF-α can modulate immune responses by affecting the expression of class I and class II MHC molecules on a variety of cell types and can stimulate many cell types to produce numerous cytokines, including IL-1, IL-6, colony-stimulating factors, and TNF-α itself.

TNF-β (also known as lymphotoxin) is genetically related to TNF-α, but is produced primarily by antigen-activated T cells (for review see Paul and Ruddle, 1988). TNF-α and TNF-β share approximately 30% amino acid residue homology, bind to the same receptors, and produce similar, but not identical, biological effects.

Biological Effects on Glial Cells

TNF-α has a diverse range of functions in the central nervous system because of its direct effects on oligodendrocytes and astrocytes. Most relevant to cen-

tral nervous system disease is the ability of TNF-α to mediate myelin and oligodendrocyte damage *in vitro* (Selmaj and Raine, 1988) and its ability to kill rat oligodendrocytes *in vitro* (Robbins et al., 1987). TNF-β exerts a more potent cytotoxic effect toward oligodendrocytes than does TNF-α, and mediates its effect by apoptosis (Selmaj et al., 1991c). This aspect of TNF-α and TNF-β activity may contribute directly to myelin damage and/or the demyelination process observed in diseases such as multiple sclerosis, EAE, and AIDS dementia complex.

TNF-α has multiple noncytotoxic effects on the astrocyte, and it may function in an autocrine fashion as astrocytes express high affinity receptors for TNF-α and secrete TNF-α upon activation (Lieberman et al., 1989; Chung and Benveniste, 1990; Velasco et al., 1991; Bethea et al., 1992a). TNF-α alone has no effect on class II MHC expression by astrocytes, but acts to enhance expression initially induced by either IFN-γ or virus (Massa et al., 1987; Vidovic et al., 1990). TNF-α acts by increasing IFN-γ-induced transcription of the class II gene, but does not affect class II mRNA stability (Vidovic et al., 1990; Panek et al., 1992). Elements within the W, X, and Y sequences of the class II MHC promoter are critical for TNF-α enhancement of IFN-γ-induced class II gene expression. TNF-α alone does not induce any nuclear proteins that bind to the class II promoter; however, combined treatment of astrocytes with both IFN-γ and TNF-α induces a DNA protein complex designated as TIC-X (TNF-α-induced complex X), which is distinct from the IFN-γ-induced nuclear protein IFNEX (Moses et al., 1992; Panek et al., 1992; Panek et al., 1994). We believe that expression of TIC-X contributes to the ability of TNF-α to enhance IFN-γ-induced class II expression in astrocytes. TNF-α has also been shown to induce proliferation of both primary bovine astrocytes (Selmaj et al., 1990) and human astroglioma cell lines (Bethea et al., 1990), thereby contributing to astrogliosis.

TNF-α is a potent inducer of cytokine production in astrocytes. Primary astrocytes and human astroglioma cell lines produce three CsFs upon stimulation with TNF-α; granulocyte-macrophage (GM)-CsF, G-CsF, and M-CsF (Malipiero et al., 1990; Aloisi et al., 1992; Lee et al., 1993). CsFs can augment inflammatory responses due to their leukocyte chemotactic properties, which would promote migration of granulocytes and macrophages to inflammatory sites within the central nervous system. TNF-α also induces IL-6 expression by both primary rat and human astrocytes (Frei et al., 1989; Aloisi et al., 1992; Norris et al., 1994). Finally, TNF-α induces expression of its own gene in rat astrocytes, suggesting a positive feedback loop for TNF-α expression (Benveniste et al., 1994).

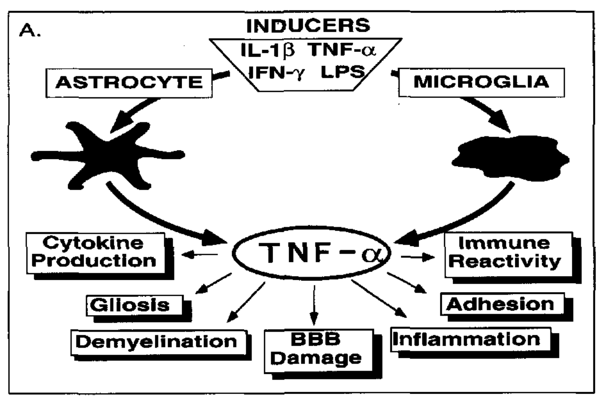
Expression by Glial Cells

Glial cells are capable of producing TNF-α upon exposure to multiple stimuli. Rat astrocytes express TNF-α in response to treatment with lipopolysaccharide (Robbins et al., 1987; Lieberman et al., 1989; Chung and Benveniste, 1990), exposure to the cytokines IFN-γ and IL-1β (Chung and Benveniste, 1990) and exposure to the neurotropic virus, Newcastle disease virus (Lieberman et al., 1989). Primary human astrocytes produce TNF-α in response to LPS (Velasco et al., 1991), and human astroglioma cell lines are capable of expressing TNF-α upon stimulation with LPS (Velasco et al., 1991) and IL-1β (Bethea et al., 1992a). Mouse microglia secrete TNF-α in response to LPS (Frei et al., 1987, Chao et al., 1992) and IFN-γ (Frei et al., 1987). These data indicate that both activated astrocytes and microglia can produce TNF-α within the central nervous system (see Figure 46-1A).

Role in Neurologic Diseases

Several groups have demonstrated that antibody to TNF-α/TNF-β can prevent the transfer of EAE by encephalitogenic T-cells in SJL/J mice (Ruddle et al., 1990; Selmaj et al., 1991b). In contrast, the disease course of EAE in SJL/J mice actively immunized with spinal cord homogenate was not affected by treatment with antibody against TNF (Teuschler et al., 1990). These differences may be due to the route of EAE induction. The above findings indicate that under certain conditions, TNF-α and TNF-β play an important role in EAE. Adding to these observations are the findings by Chung et al. (1991) that astrocytes from EAE-susceptible and -resistant rat strains differ in their ability to express TNF-α protein. Astrocytes from Lewis rats (susceptible) produce TNF-α in response to IFN-γ/IL-1β, while astrocytes from Brown Norway rats (resistant) do not. The capacity for TNF-α production by Lewis astrocytes, especially in response to disease-related cytokines such as IFN-γ and IL-1, may contribute to disease susceptibility and to the inflammation and demyelination associated with EAE.

In vivo studies suggest that TNF-α/TNF-β may be involved in multiple sclerosis. TNF-α-positive astrocytes and macrophages have been identified in the brains of multiple sclerosis patients, particularly in the plaque region (Hofman et al., 1989). Selmaj et al. (1991a) have determined that both TNF-α and



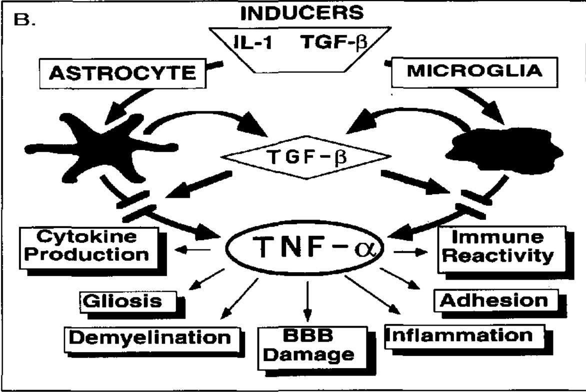


FIG. 46-1. (A) Effects of TNF-α within the central nervous system (CNS). TNF-α can be produced by both astrocytes and microglia in response to various inducers (IL-1, IFN-γ, TNF-α, LPS), and has a number of effects on glial cells, endothelial cells, and immune cells found both within the CNS and/or the CNS vasculature. (B) Effects of TGF-β within the CNS. TGF-β can be produced by activated astrocytes, microglia, and oligodendrocytes (*not shown*) in response to various inducers, including IL-1 and TGF-β. TGF-β can act in an autocrine or paracrine manner to inhibit TNF-α production, gliosis, demyelination, and inflammation within the CNS.

TNF-β are present in multiple sclerosis plaque regions, and that TNF-α is localized within astrocytes, whereas TNF-β is associated with microglia and T cells. Increased cerebrospinal fluid levels of TNF-α have been documented in patients with multiple sclerosis (Maimone et al., 1991; Sharief et al., 1991). Sharief et al. (1993, 1992) then went on to demonstrate that a strong correlation exists between cerebrospinal fluid levels of TNF-α, disruption of the blood-brain barrier, cerebrospinal fluid pleocytosis, and increased levels of circulating intercellular adhesion molecules-1 in both the serum and cerebrospinal fluid of patients with active multiple sclerosis. Other studies have failed to demonstrate TNF-α in the cerebrospinal fluid of multiple sclerosis patients (Gallo et al., 1989). The failure to detect TNF-α may be due to differences in the patients examined, or to the instability of TNF-α in cerebrospinal fluid if not treated with a protease inhibitor. Additionally, TNF-α may only be present locally in the central nervous system during different stages of multiple sclerosis and not detectable in cerebrospinal fluid.

Elevated levels of TNF-α have been demonstrated in the cerebrospinal fluid of AIDS patients, and TNF-α staining in brains from AIDS patients localizes with some endothelial cells and astrocytes, but mostly with macrophages/microglia (Tyor et al., 1992). TNF-α has been shown to activate and enhance HIV-1 replication in macrophages (Poli et al., 1990b), and thus may contribute to the pathogenesis of AIDS dementia complex. Astrocytes and microglia have a direct role in this process, as TNF-α produced by these cells induces the expression of HIV-1 in macrophages (Vitkovic et al., 1990; Peterson et al., 1992).

INTERLEUKIN-6 AND GLIAL CELLS

IL-6, similar to IL-1 and TNF-α, is a pleiotropic cytokine involved in the regulation of inflammatory and immunologic responses (for review see van Snick, 1990). IL-6, a 26,000 Dalton molecule, is secreted by a wide range of activated cells, including fibroblasts, monocytes, B cells, endothelial cells, T cells, microglia, and astrocytes. The two best described functions of IL-6 are on hepatocytes and B cells. IL-6 stimulates hepatocytes to synthesize several plasma proteins, such as fibrinogen and C-reactive protein, which contribute to the acute phase response. IL-6 serves as the principal cytokine for inducing terminal differentiation of activated B cells into immunoglobulin-secreting plasma cells.

Biological Action on Glial Cells

IL-6 has a mitogenic effect on bovine astrocytes (Selmaj et al., 1990), which may contribute to astrogliosis. Astrocytes respond to IL-6 by secreting nerve growth factor, which induces neural differentiation (Frei et al., 1989). IL-6 has been demonstrated to inhibit TNF-α production by monocytes (Aderka et al., 1989). Since astrocytes can secrete TNF-α, and TNF-α induces IL-6 production by astrocytes (see below), IL-6 may be involved in the negative regulation of TNF-α expression in the central nervous system.

Expression by Glial Cells

IL-6 is produced within the central nervous system by both astrocytes and microglia. Primary human, mur-

ine, and rat astrocytes can secrete IL-6 in response to a number of stimuli, including virus, IL-1, TNF-α, IFN-γ plus IL-1, lipopolysaccharide, calcium ionophore, and norepinephrine (Frei et al., 1989; Lieberman et al., 1989; Benveniste et al., 1990; Aloisi et al., 1992; Norris and Benveniste, 1993). Mouse microglia secrete IL-6 upon infection with virus or stimulation with the cytokine macrophage colony-stimulating factor (Frei et al., 1989), and rat microglia express IL-6 upon stimulation with lipopolysaccharide (Norris and Benveniste, 1993).

The molecular mechanism(s) by which IL-1β and TNF-α activate IL-6 expression have been examined by transient transfection of the IL-6 promoter linked to a reporter gene in primary rat astrocytes. Both IL-1β and TNF-α act at the transcriptional level to induce IL-6 gene expression, and use of deletion mutants revealed that the nuclear factor-kappa B (NF-κB)-like binding site is required for cytokine induction of IL-6 promoter activity (Sparacio et al., 1992). Nuclear proteins isolated from IL-1β- or TNF-α-treated astrocytes are specific for the NF-κB-like binding site within the IL-6 promoter, and it appears that the action of IL-1β and TNF-α is mediated by post-translational activation of preexisting cytoplasmic NF-κB.

Role in Neurologic Diseases

Increased IL-6 levels have been found in the cerebrospinal fluid of mice suffering acute EAE, and Gijbels et al. (1990) suggest that local production of IL-6 is responsible, since serum levels of IL-6 were not elevated. IL-6 mRNA levels increase rapidly during acute EAE in SJL/J mice, and decline when clinical symptoms resolve (Kennedy et al., 1992). In contrast, IL-6 protein is not found in the central nervous system of SJL/J mice which acquire EAE through MBP peptide immunization (Merrill et al., 1992). Again, the different methods of immunization and analysis may account for the conflicting results.

One of the hallmarks of multiple sclerosis is intrathecal B cell activation as evidenced by elevation of the cerebrospinal fluid IgG index and the presence of oligoclonal IgG bands in the cerebrospinal fluid (Tourtellotte and Ma, 1978). Since IL-6 is involved in differentiation of B cells into immunoglobulin-secreting plasma cells, there has been interest in determining whether elevated IL-6 levels could be responsible for local B-cell responses within the central nervous system. Results have been conflicting; two groups report that IL-6 is not detected in multiple sclerosis cerebrospinal fluid (Frei et al., 1988; Houssiau et al., 1988), while two more recent studies suggest that IL-6 is elevated in multiple sclerosis cerebrospinal fluid (Maimone et al., 1991), and multiple sclerosis plasma (Frei et al., 1991). These latter findings suggest that there is a heightened systemic B-cell response in multiple sclerosis.

Elevated central nervous system IL-6 levels have been documented in AIDS dementia complex patients (Tyor et al., 1992). IL-6 has been shown to upregulate production of HIV in infected cells of the monocytic lineage, and to act synergistically with TNF-α (Poli et al., 1990a). IL-6 produced by human astrocytes can stimulate HIV-1 expression in a promonocyte cell line (Vitkovic et al., 1991), thus, intracerebral production of IL-6 by astrocytes may contribute to HIV replication within the central nervous system.

COLONY-STIMULATING FACTORS AND GLIAL CELLS

The group of cytokines that have potent stimulatory effects on the growth and differentiation of bone marrow progenitor cells are collectively called CsFs. By stimulating the growth and differentiation of bone marrow cells, CsFs act to provide inflammatory leukocytes (for review see Golde and Gasson, 1988). Interleukin-3 (IL-3), also known as multi-CsF, is produced by T-helper cells, and acts on the most immature bone marrow progenitors to induce the expansion of cells that differentiate into all known mature cell types. Granulocyte-macrophage colony-stimulating factor (GM-CsF) is produced by a number of activated cells, including T cells, macrophages, endothelial cells, fibroblasts, and astrocytes. GM-CsF acts on bone marrow progenitor cells already committed to differentiate into granulocytes and monocytes. GM-CsF can also interact with various mononuclear phagocytes, including microglia, to induce their activation. Macrophage colony-stimulating factor (M-CsF), also called CsF-1, is made by macrophages, endothelial cells, and fibroblasts. M-CsF acts primarily on progenitor cells already committed to develop into monocytes; these progenitor cells are more mature than the targets for GM-CsF. Granulocyte colony-stimulating factor (G-CsF) is made by the same cells that produce GM-CsF, and acts primarily on bone marrow progenitors already committed to develop into granulocytes.

Biological Action on Glial Cells

As activation of microglia, the macrophage of the brain, is an important early response to brain

trauma, there has been interest in how the activation and differentiation of microglia is induced. Frei et al. (1987) demonstrated that both IL-3 and GM-CsF induced murine microglia to proliferate. Further *in vitro* studies by Giulian and Ingeman (1988) also showed that rat microglia could proliferate in response to IL-3, GM-CsF, and M-CsF, and that both IL-3 and GM-CsF induced more rapid phagocytosis by microglia. They also performed *in vivo* experiments in which recombinant GM-CsF, IL-3, M-CsF, or G-CsF was infused into the cerebral cortex of rats. Both GM-CsF and IL-3 stimulated the appearance of microglia at the site of injection, and the phagocytic capability of these cells. M-CsF has also been shown to induce microglia to produce IL-6 (Frei et al., 1989), and inhibits both basal and IFN-γ-induced class II MHC expression on microglia (Lee et al., 1993). These findings indicate that some of the CsFs can enhance inflammatory responses within the central nervous system by activation of microglia.

Expression by Glial Cells

Astrocytes appear to be the major source of CsFs within the brain. Unstimulated astrocytes do not constitutively express GM-CsF and G-CsF, but are induced to by both TNF-α and LPS (Malipiero et al., 1990; Ohno et al., 1990). Primary human astrocytes were recently shown to produce GM-CsF, M-CsF, and G-CsF in response to both IL-1 and TNF-α (Aloisi et al., 1992; Lee et al., 1993). Unstimulated human astrocytes constitutively expressed mRNA for M-CsF, but had to be induced by IL-1 or TNF-α to express transcripts for GM-CsF or G-CsF. Similar observations have been made for murine astrocytes (Hao et al., 1990; Thery et al., 1990). Microglia can be induced to express mRNA for both M-CsF and G-CsF upon stimulation with LPS (Malipiero et al., 1990; Thery et al., 1990), and human fetal microglia constitutively express M-CsF mRNA and protein, which is enhanced by LPS (Lee et al., 1993).

TRANSFORMING GROWTH FACTOR-BETA AND GLIAL CELLS

TGF-β is a dimeric protein of approximately 28,000 Daltons that is synthesized by almost all cell types. It is normally secreted in a latent form that must be activated by proteases (for review see Massague, 1990). The TGF-β family is comprised of five different isoforms encoded by separate genes, which have high amino acid sequence homology. The actions of TGF-β are highly pleiotropic and include inhibiting the proliferation of many cell types (epithelial, endothelial, lymphoid, and hematopoietic cells), promoting the growth of new blood vessels (angiogenesis), serving as a chemotactic factor for macrophages, and inhibiting immune and inflammatory responses. TGF-β has been demonstrated to inhibit the production of numerous cytokines and is thought to function as a negative regulator of immune responses.

Biological Action on Glial Cells

TGF-β can modulate the activity of astrocytes, microglia, and oligodendrocytes. TGF-β1 and TGF-β2 can inhibit IFN-γ-induced class II MHC expression on rat astrocytes (Schluesener, 1990), inhibit proliferation of rat astrocytes (Lindholm et al., 1992; Morganti-Kossmann et al., 1992), and can act as chemotactic agents for both rat astrocytes and microglia (Yao et al., 1990; Morganti-Kossmann et al., 1992). TGF-β inhibits TNF-α production by microglia (Suzumura et al., 1993), and astrocytes (Benveniste et al., 1994). TGF-β affects oligodendrocytes by promoting differentiation of these cells (McKinnon et al., 1993). Since TGF-β is produced by glial cells (see below), locally produced TGF-β may contribute to the recruitment and activation of glial cells (both astrocytes and microglia) at local inflammatory sites within the central nervous system.

Expression by Glial Cells

Primary rat astrocytes, upon exposure to exogenous TGF-β, secrete TGF-β (Wahl et al., 1991). This same group later demonstrated that rat astrocytes constitutively express mRNA for TGF-β1, which is increased upon exposure to exogenous TGF-β1, indicating that TGF-β1 levels can be regulated in an autocrine manner (Morganti-Kossmann et al., 1992). da Cunha and Vitkovic (1992) have also shown that primary rat astrocytes constitutively express mRNA for TGF-β1, but do not constitutively secrete TGF-β1 protein. However, upon stimulation with IL-1, the astrocytes secrete TGF-β1. Further work from the Vitkovic laboratory has demonstrated that both primary rat oligodendrocytes and microglia can secrete TGF-β1 upon stimulation with IL-1α (da Cunha et al., 1993). Taken together, the above studies indicate that all three glial cell types, astrocytes, oligodendrocytes, and microglia, are capable of secreting TGF-β. As TGF-β exerts many immunosuppressive effects,

TGF-β produced by glial cells may act to restrict and/or downregulate inflammatory processes within the central nervous system. Additionally, TGF-β may exert beneficial effects on oligodendrocytes by inducing differentiation of these cells.

Role in Neurologic Diseases

Several studies have demonstrated that TGF-β improves the clinical course of EAE in SJL/J mice (Johns et al., 1991; Kuruvilla et al., 1991; Racke et al., 1991). Injection of TGF-β1 could delay development of EAE, and prevent the incidence of relapse in these mice (Kuruvilla et al., 1991). Racke et al. (1991) demonstrated that TGF-β1 inhibited the activation of MBP-specific lymph node cells *in vitro,* which reduced that capacity of these cells to transfer EAE. Additionally, injection of TGF-β1 resulted in an improved clinical course, even when administered during ongoing disease. Johns et al. (1991), showed that *in vivo* injection of TGF-β1 reduced the incidence of clinical disease, as well as the severity of inflammation and demyelination within the central nervous system. In addition, TGF-β1, -β2, and -β3 were present in inflammatory lesions within the brain. Santambrogio et al. (1993) examined the mechanism(s) by which TGF-β modulates the disease course of EAE. Their results demonstrated that TGF-β treatment prevented the accumulation of myelin-sensitized T cells in brain and spinal cord, indicating the protective effect of TGF-β is exerted at the level of the central nervous system and/or its vascular endothelium. It was further suggested that TGF-β may act by inhibiting both the production of TNF-α and its effects within the central nervous system, including the involvement of TNF-α in inflammatory processes (see Figure 46-1B).

TGF-β1 has been identified in the brains of patients with AIDS, but not in control brain tissue (Wahl et al., 1991). The TGF-β staining was localized to macrophages, microglia, and astrocytes, especially in areas of diseased brain. Moreover, HIV-1-infected monocytes secreted a factor that induced cultured astrocytes to secrete TGF-β, which in all likelihood is TGF-β itself. TGF-β has bifunctional effects on HIV expression, either inhibiting or enhancing replication, depending on the time of exposure (Lazdins et al., 1991; Poli et al., 1991). Thus, HIV-1-induced TGF-β production by macrophages may act in an autocrine manner to modulate HIV replication, or in a paracrine fashion to induce astrocytes to produce TGF-β. By either pathway, TGF-β may play an important role as a regulator of HIV expression in infected macrophages/microglia.

CHEMOATTRACTANT CYTOKINES AND GLIAL CELLS

A family consisting of at least 10 distinct novel 8000 to 10,000 Dalton cytokines has been described recently. Collectively designated as "chemokines" or "intercrines," these cytokines are the product of two related gene families, members of which exhibit sequence homology and structural similarities (for review see Oppenheim et al., 1991). These cytokines are expressed locally in response to inflammatory stimuli, and act to recruit inflammatory cells via their chemoattractant properties. In the mouse, representative chemokines include JE/MCP-1 and IP-10.

A recent study by Ransohoff et al. (1993) investigated chemokine production in the central nervous system during EAE to identify factors potentially governing inflammatory cell accumulation during immune-mediated demyelination. EAE was induced in SJL/J mice using PLP peptides, and expression of JE/MCP-1 and IP-10 mRNA was examined by RT-PCR and *in situ* hybridization. Astrocytes were the only cells in the central nervous system that expressed mRNA transcripts for JE/MCP-1 and IP-10, and expression correlated with the appearance of clinical and histologic EAE. *In vitro* studies support this finding, since the human astroglioma cell line U-105MG constitutively secretes high amounts of JE/MCP-1 (Yoshimura et al., 1989), and human astrocytic cells express IP-10 mRNA following exposure to IFN-γ and TNF-α (Ransohoff et al., 1993). These findings suggest that astrocytes play a significant role in the accumulation of inflammatory cells within the central nervous system due to their ability to secrete "chemokines."

SUMMARY

This chapter has summarized studies showing that cells of the immune system and glial cells of the central nervous system use many of the same cytokines as communication signals. Activated astrocytes and microglia are the principal sources of these cytokines (IL-1, IL-6, TNF-α, CsFs, TGF-β, chemokines), although oligodendrocytes are capable of expressing IL-1 and TGF-β. The glial cells respond to these cytokines by changes which include the following (see Table 46-1 for summary):

1. Regulation of proliferation and differentiation
2. Induction, enhancement, and inhibition of the expression of cell surface antigens (class I MHC, class II MHC)
3. Stimulation of the secretion of other cytokines

TABLE 46–1. *Cytokines in the Central Nervous System*

Glial Cell Type	Cytokines Produced	Response to Cytokines							
		IFN-γ	IL-1	TNF-α/β	IL-6	GM-CsF	M-CsF	IL-3	TGF-β
Astrocyte	IL-1 TNF-α IL-6 M-CsF G-CsF GM-CsF TGF-β Chemokines	Increases class I MHC Induces class II MHC Increases TNF-α receptors Primes cells for TNF-α, IL-6 production	Proliferation IL-6 production TNF-α production G-CsF, M-CsF, GM-CsF production TGF-β production	Increases class I MHC Enhances class II MHC Proliferation IL-6 production G-CsF, M-CsF, GM-CsF production TNF-α production	Proliferation NGF production	?	No effect; do not have M-CsF receptors	?	Chemotactic Inhibits proliferation Inhibits class II MHC Inhibits GM-CsF production
Microglia	IL-1 TNF-α IL-6 M-CsF G-CsF TGF-β	Increases class I MHC Induces class II MHC TNF-α production	TGF-β production	Inhibits class II MHC	?	Proliferation Increases phagocytosis	Proliferation Morphological changes IL-6 production Inhibits class II MHC	Proliferation Increases phagocytosis Morphological changes	Chemotactic
Oligodendrocyte	IL-1 TGF-β	Increases class I MHC	TGF-β production	Cell death Myelin damage	?	?	?	?	Differentiation of cells

This is by no means a complete listing since cytokines affect the expression of other genes by glial cells such as complement components, ICAM-1, and Fc receptors, which were not discussed in this chapter. There is a complex circuitry of interactions mediated by cytokines, especially in the event of blood-brain barrier damage and lymphoid/mononuclear cell infiltration into the central nervous system. The secretion of IFN-γ by infiltrating activated T cells may be the initiating signal for glial cell activation by inducing astrocytes and microglia to express class I and II MHC antigens, and priming these cells for subsequent cytokine production. In addition, infiltrating activated macrophages produce cytokines such as IL-1, TNF-α, and IL-6, which in concert with IFN-γ would trigger glial cells to produce their own cytokines. The activation of astrocytes and microglia to secrete cytokines may contribute to the propagation of intracerebral immune and inflammatory responses initiated by immune cells. The cytokine cascades ongoing in the central nervous system could ultimately be downregulated due to the presence of immunosuppressive cytokines such as TGF-β and IFN-β, or perpetuated, leading to disease progression. Whether immune and inflammatory responses within the central nervous system are propagated or suppressed depends on a number of parameters including:

1. The activational status of these cells
2. Cytokine receptor levels on glial and immune cells
3. The concentration and location of cytokines in the central nervous system
4. The temporal sequence in which a particular cell is exposed to numerous cytokines

Many of the studies cited in this chapter reflect the capacity of cultured primary murine, rodent, and human cells or human glial cell lines to function *in vitro*. These studies provide important information on how these cells can respond to a variety of stimuli, and provide useful working models for further understanding the nature of the second messenger signals utilized by glial cells, delineating *cis*-acting DNA regulatory elements and nuclear proteins involved with MHC and cytokine expression by glial cells, and characterizing the types of cytokine receptors found on glial cells. The *in vitro* studies suffer, however, from the fact that the cells have been taken out of their natural microenvironment. *In vivo* studies can address the capacity of cells to express cytokines or surface antigens in their normal setting, or in the case of EAE or multiple sclerosis during ongoing disease. Many of the *in vivo* studies have demonstrated that particular glial cells can express certain genes, which correlate with *in vitro* results. However, there still is controversy in the literature regarding production of cytokines by astrocytes, oligodendrocytes, and microglia. Some of the *in vivo* studies suggest that microglia are the predominant source of some cytokines such as IL-1 and TNF-α. One of the problems has been that most of the *in vivo* studies have examined cytokine *protein* expression within the central ner-

vous system. These studies cannot distinguish between cells producing cytokines or binding them via specific receptors. *In situ* hybridization to examine cytokine mRNA expression coupled with immunohistochemistry to identify the cell type producing the cytokine will definitively address the issue of which endogenous glial cells produce cytokines *in vivo*. Thus, the combined use of both *in vitro* and *in vivo* systems, and extrapolation between the two, will provide an understanding of the mechanism(s) by which cytokines influence glial cell function.

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X Neuron-Glial Cell Interactions

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47 Glial cell regulation of extracellular potassium

ERIC A. NEWMAN

Neurons in the central nervous system are maintained in a highly regulated environment. The blood-brain barrier provides an effective shield that isolates the brain from variations in blood levels of organic and inorganic molecules. This isolation is essential as neurons are extremely sensitive to such common ions as K^+ and Ca^{2+} and to amino acids, including glutamate and γ-aminobutyric acid (GABA). The blood-brain barrier by itself cannot ensure a stable extracellular environment, however. Neuronal activity results in the release of neurotransmitters and in extracellular pH shifts, both of which modify neuronal function.

Neuronal activity also results in the release of K^+ and its accumulation within extracellular space. Activity-dependent increases in extracellular K^+ concentration ($[K^+]_o$) would rapidly lead to substantial changes in neuronal behavior if these increases were not buffered. The central nervous system possesses several homeostatic mechanisms that help to regulate activity-dependent $[K^+]_o$ variations. Glial cells play a major role in this regulatory process.

Although the discussion of K^+ regulatory mechanisms in this chapter is limited to vertebrate species, glial cells of invertebrates also help regulate $[K^+]_o$ (Coles and Tsacopoulos, 1979; Gardner-Medwin et al., 1981; Coles, 1989; Wuttke, 1990).

ACTIVITY-DEPENDENT VARIATIONS IN $[K^+]_o$

The resting level of $[K^+]_o$ in extracellular space is low, approximately 3 mM. Thus, the release of even small amounts of K^+ from active neurons leads to a substantial percentage increase in $[K^+]_o$ (Adelman and Fitzhugh, 1975; Coles and Poulain, 1991). Neurons at rest release little K^+. Potassium efflux increases greatly, however, when neurons depolarize, due to the increased electrical driving force on K^+ and to the opening of voltage-gated K^+ channels.

Ion-Selective Microelectrode Measurements of $[K^+]_o$ Variations

Cerebral Cortex. Activity-dependent variations in $[K^+]_o$ in the central nervous system have been measured directly with ion-selective microelectrodes. Measurements reveal that normal physiological stimuli result in modest $[K^+]_o$ increases in the cortex, which rarely exceed 1 mM (Figure 47-1A; (Kelly and Van Essen, 1974; Singer and Lux, 1975). Much larger increases are seen, however, under pathological conditions. Increases to 8 to 12 mM are seen during episodes of epileptic discharge (Lux, 1974; Lothman et al., 1975), while electrical stimulation results in $[K^+]_o$ increases, which can rise as high as 12 mM (Somjen, 1979) (Figure 47-2). An intriguing aspect of these $[K^+]_o$ increases is that $[K^+]_o$ does not (usually) exceed a ceiling level of ~12 mM, no matter how intense the electrical stimulation or the epileptic activity (Heinemann and Lux, 1977). A ceiling level of ~10 mM is also seen in the optic nerve, a white matter tract (Connors et al., 1982). The existence of this ceiling $[K^+]_o$ level is direct evidence that the central nervous system possesses powerful mechanisms for removing K^+ from extracellular space.

Retina. Activity-dependent variations in $[K^+]_o$ have been extensively characterized in the vertebrate retina (Figure 47-1B). Light stimulation results in $[K^+]_o$ increases in the two synaptic layers of the retina, an increase of less than 1 mM in the inner plexiform layer, and a smaller more transient increase in the outer plexiform layer (Karwoski and Proenza, 1977; Dick and Miller, 1985; Karwoski et al., 1985). In addition, photoreceptor hyperpolarization generates a large light-evoked $[K^+]_o$ decrease in the distal retina, which can reach several millimolar under optimal conditions (Oakley and Green, 1976).

Effect of $[K^+]_o$ Variations on Neuronal Function

The resting membrane potential of neurons reflects, in large part, the K^+ equilibrium (Nernst) potential

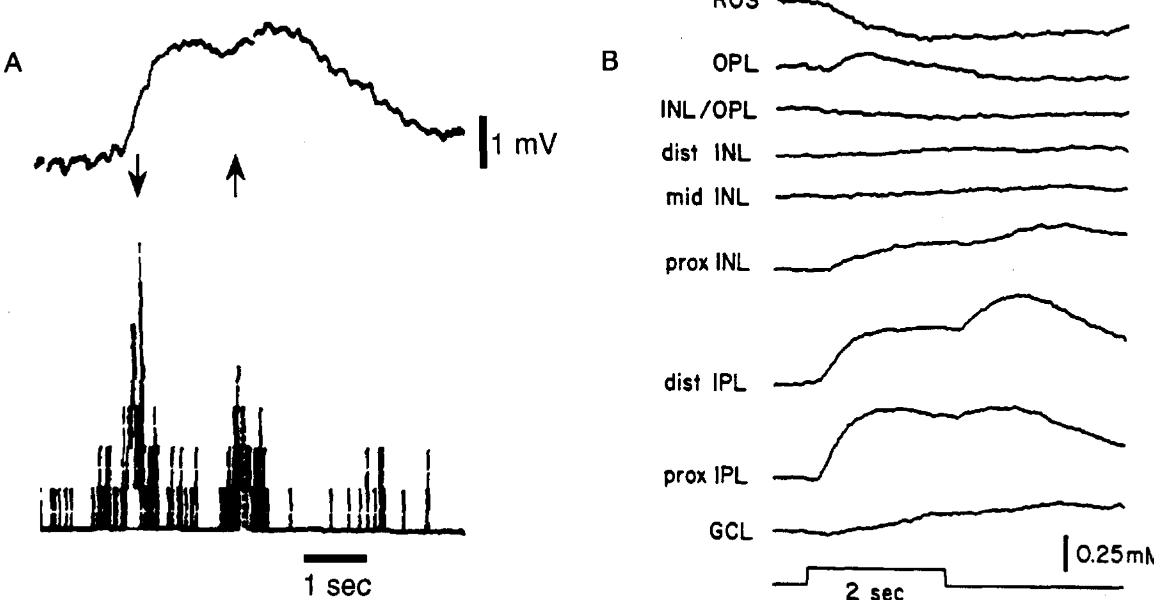

FIG. 47-1. Variations in $[K^+]_o$ within the central nervous system evoked by sensory stimuli. (**A**) *Upper trace:* $[K^+]_o$ variations in the cat visual cortex evoked by movement of a bar down (↓) and then up (↑) through the receptive field of a hypercomplex cell. *Lower trace:* Activity of an adjacent neuron, recorded with the reference barrel of the double-barreled ion selective microelectrode. The 1-mV calibration bar for $[K^+]_o$ corresponds to ~0.17 mM. [Modified from Singer and Lux (1975) with permission.] (**B**) $[K^+]_o$ variations in the frog retina (retinal slice preparation) evoked by a diffuse 2-second light flash. $[K^+]_o$ increases occur in the inner plexiform layer (*IPL*) and the outer plexiform layer (*OPL*), while a $[K^+]_o$ decrease occurs in the subretinal space (rod outer segment layer, *ROS*). [From Karwoski et al. (1985), with permission.]

which, in turn, is determined by the K^+ concentration gradient across the cell membrane. Physiological increases in $[K^+]_o$ result in small but significant cell depolarizations which can influence neuronal function in many ways, including modification of the threshold for action potential initiation and modulation of voltage-sensitive receptor processes (e.g., the *N*-methyl-D-aspartate (NMDA) receptor). Synaptic transmission is particularly sensitive to $[K^+]_o$ variations. An increase in $[K^+]_o$ beyond 5 mM in hippocampal slices, for instance, leads to a reduction in the efficacy of synaptic transmission (Rausche et al., 1990).

Variations in $[K^+]_o$ as Possible Signals

Activity-dependent variations in $[K^+]_o$ are normally viewed as perturbations of the neuronal microenvi-

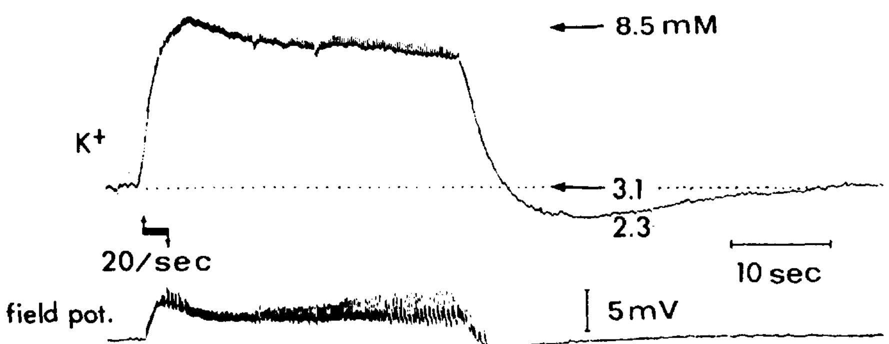

FIG. 47-2. Variations in $[K^+]_o$ in the cat cerebral cortex evoked by electrical stimulation of the cortical surface. *Upper trace:* $[K^+]_o$. *Lower trace:* The field potential (recorded with the reference barrel of the ion-selective microelectrode, negative upward). The brief electrical stimulus (*horizontal bar between traces*) triggers a prolonged epileptiform discharge and $[K^+]_o$ increase. The electrical stimulation ends before the peak of the $[K^+]_o$ response. Note that $[K^+]_o$ drops below control level (*dotted line*) following the end of the neuronal activity. [From Lux (1974), with permission.]

ronment which must be minimized in order to preserve normal brain function. It is possible, however, that variations in $[K^+]_o$ also serve as signals between neurons or between neurons and other cells (Orkand et al., 1966). The magnitude of a $[K^+]_o$ increase is a good index of overall neuronal activity, and thus conveys important information. Increases in $[K^+]_o$ may serve as signals mobilizing energy sources for neurons, perhaps from glial cells (see Chapter 52). Potassium variations may also constitute a signal which couples increases in neuronal activity to localized increases in blood flow (Paulson and Newman, 1987).

OVERVIEW OF POTASSIUM REGULATORY MECHANISMS

There are several mechanisms which aid in the regulation of activity-dependent variations in $[K^+]_o$ (Figure 47-3). The simplest of these is diffusion of K^+ through extracellular space. Extracellular $[K^+]$ can also be regulated by K^+ uptake into cells, either neurons or glial cells. Both active uptake, mediated by Na^+/K^+ ATPases, or passive uptake, mediated by flux through transporters or channels, operate to reduce $[K^+]_o$ increases. Glial cells accumulate K^+ at a far higher rate than do neurons (Walz and Hertz, 1982), indicating the importance of these cells to K^+ regulation.

Variations in $[K^+]_o$ can also be regulated by K^+ transport via current flow through glial cells (Orkand et al., 1966). This process, termed "K^+ spatial buffering," leads to the transfer of extracellular K^+ from regions where $[K^+]_o$ is higher to regions where K^+ is lower. See also Chapter 26. In a specialized form of spatial buffering, termed "K^+ siphoning" (Newman et al., 1984), K^+ currents are selectively directed out from the endfeet of glial cells.

DIFFUSION

Activity-dependent increases in $[K^+]_o$ are dissipated, in part, by simple diffusion of K^+ through extracellular space. The movement of ions, including K^+, in extracellular space is accurately described by diffusion with the ion diffusion coefficient, D (~$2 \cdot 10^{-5}$ $cm^2\ s^{-1}$ for K^+) reduced by a factor λ^2, where λ is the tortuosity of the diffusional path through extra-

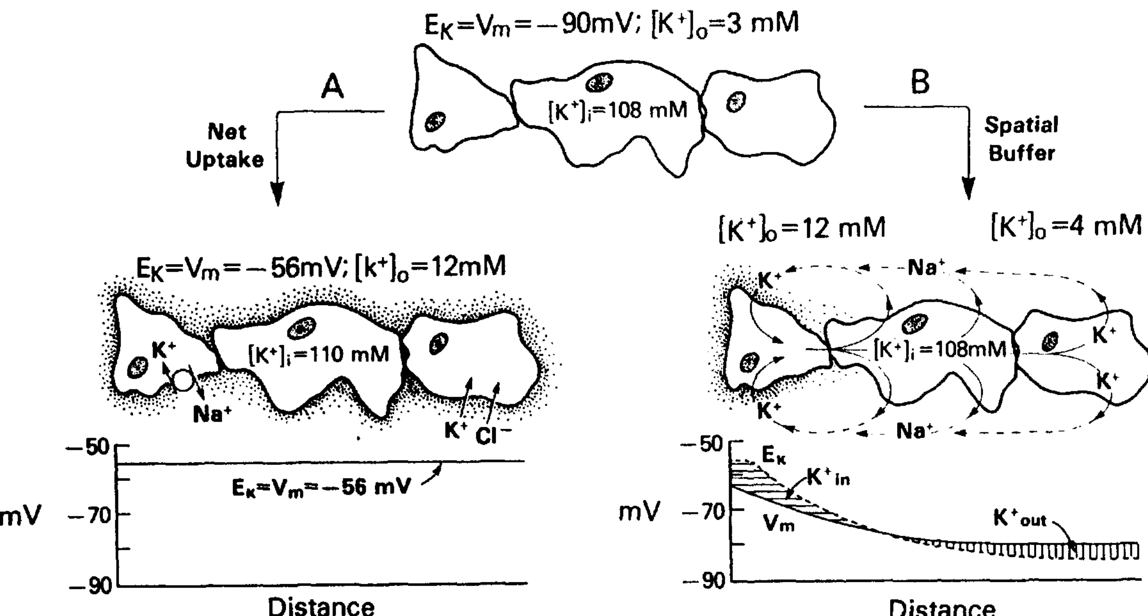

FIG. 47-3. Overview of glial cell K^+ regulatory mechanisms. Top: Three glial cells, coupled by gap junctions. Left: Net K^+ accumulation. When $[K^+]_o$ is raised uniformly (*shaded area surrounding cells*) uptake mechanisms lead to K^+ accumulation and to an increase in $[K^+]_i$ in the glial cells. Uptake occurs by active pumping (*left cell*) and by passive uptake (*right cell;* $Na^+/K^+/2Cl^-$ transport or $K^+ + Cl^-$ flux driven by Donnan forces). Increased $[K^+]_o$ results in cell depolarization (*shown in graph below cells*). **Right:** K^+ transfer via spatial buffer current. When $[K^+]_o$ is raised locally (*shaded area surrounding left cell*) a current flow is established with K^+ entering the left cell and K^+ leaving the other two cells. This current results in the transfer of K^+ from the shaded region where $[K^+]_o$ is raised to regions where $[K^+]_o$ is lower. The graph below the cells illustrates that the transmembrane K^+ fluxes are driven by the difference between the cell membrane potential (V_m) and the K^+ equilibrium potential (E_K). K^+ influx occurs when E_K is positive to V_m (*left cell*). K^+ efflux occurs when V_m is positive to E_K (*middle and right cells*). Although *not illustrated,* net K^+ accumulation would also occur in the left cell. [From Orkand (1986), with permission.]

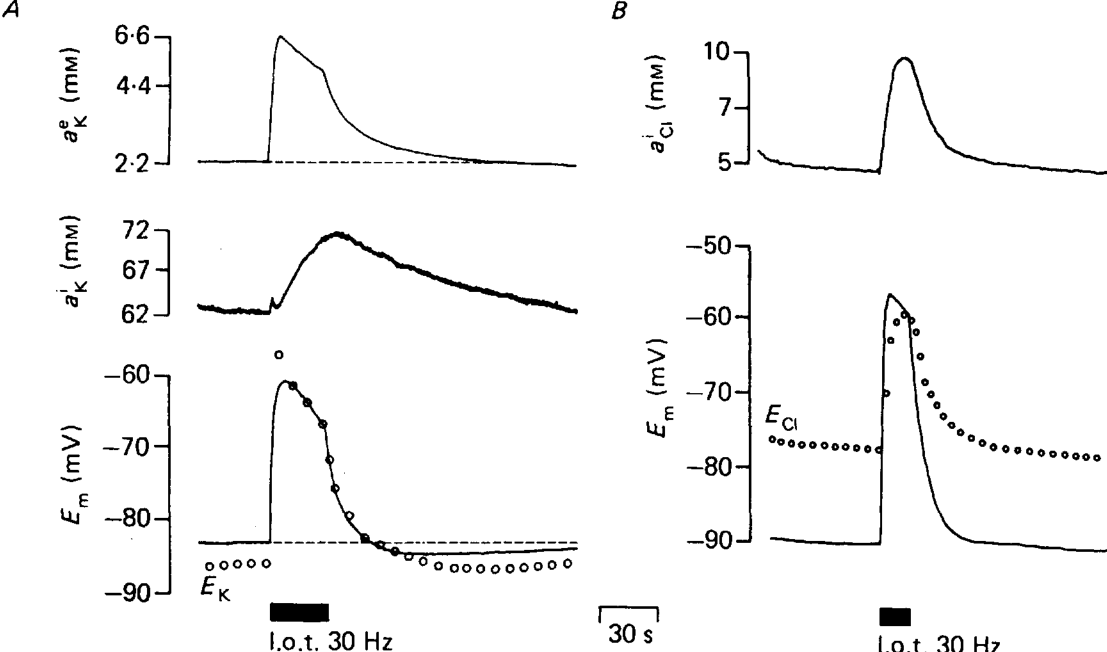

FIG. 47-4. Accumulation of K^+ and Cl^- in glial cells of the guinea pig olfactory cortex (slice preparation). (A) Electrical stimulation of the lateral olfactory tract (*l.o.t.*) results in an increase in $[K^+]_o$ (*top*), an increase in $[K^+]_i$ (*middle*), and to cell depolarization (*bottom*). (B) Stimulation also results in an increase in $[Cl^-]_i$ (*top*). The K^+ accumulation shown in Figure A was due partially to a Ba^{2+}-sensitive $K^+ + Cl^-$ influx and partially to ouabain-sensitive active uptake. Ion activities, rather than concentrations, are shown and were measured with intracellular ion selective microelectrodes. [From Ballanyi et al. (1987), with permission.]

cellular space (Chapter 26, this volume; Nicholson and Phillips, 1981). The effective diffusion coefficient, D/λ^2, equals ~$8 \cdot 10^{-6}$ cm^2 s^{-1} for K^+.

ACTIVE POTASSIUM UPTAKE

Potassium Accumulation

Activity-dependent $[K^+]_o$ increases are regulated by active K^+ uptake into cells (Figure 47-3A). All the K^+ released by active neurons must eventually be returned to these cells. This uptake occurs primarily by the action of the neuronal Na^+/K^+ ATPase (Ballanyi et al., 1984; see also Chapters 16 and 18). Evidence suggests that uptake occurs slowly in neurons, over minutes following tetanic stimulation (Heinemann and Lux, 1975; Ballanyi et al., 1984). Thus, active uptake by neurons is not sufficient to buffer rapid $[K^+]_o$ increases. This conclusion is underlined by the results of a study on cultured cells, which demonstrated that active uptake in neurons was fivefold lower than was uptake in glial cells (Walz and Hertz, 1982).

Glial cells also possess a Na^+/K^+ ATPase that is believed to play an important role in removing excess K^+ from extracellular space. The properties of the glial Na^+ pump differ from those of the neuronal pump in its activation by external K^+ (see Chapter 16). Whereas the neuronal pump saturates as $[K^+]_o$ reaches 3 to 5 mM, the activity of the glial pump continues to rise until $[K^+]_o$ reaches 18 to 20 mM (Franck et al., 1983). Thus, the glial Na^+/K^+ ATPase is well suited to maintaining $[K^+]_o$ near 3 mM and will operate efficiently to pump K^+ when $[K^+]_o$ is raised to abnormally high levels (Franck et al., 1983; Reichenbach et al., 1987).

Active glial cell uptake of K^+ has been demonstrated directly in ion-selective microelectrode experiments on guinea pig olfactory cortical slices (Ballanyi et al., 1987) (Figure 47-4). Following electrical stimulation, the intracellular K^+ concentration ($[K^+]_i$) of glial cells in slices increased substantially. Application of the Na^+/K^+ ATPase inhibitor ouabain decreased this K^+ rise, indicating that the K^+ accumulation was due, in part, to active uptake.

Active K^+ uptake has also been described in studies on cultured glial cells. In cultured mouse astrocytes, increased $[K^+]_o$ led to a substantial K^+ accumulation (measured by ^{42}K uptake), approximately half of which was ouabain-sensitive (Walz and Hinks, 1985). Similar findings have been reported for cultured oligodendrocytes (Kettenmann et al., 1987; Ballanyi and Kettenmann, 1990).

Activity-dependent increases in $[K^+]_o$ in the brain are often followed by a postactivity K^+ undershoot in which $[K^+]_o$ falls below prestimulus levels (Figure

47-2; Heinemann and Lux, 1975; Heinemann and Lux, 1977). This decrease can last for several minutes following intense neuronal activity. The $[K^+]_o$ undershoot is believed to be generated by active uptake of K^+ by neurons previously activated (Ballanyi et al., 1984), perhaps stimulated by excess internal Na^+ in these cells. This $[K^+]_o$ depletion may play an important role in $[K^+]_o$ regulation in that it provides a driving force for the restoration of K^+, temporarily stored in glial cells or transported by spatial buffering currents, back to the activated neurons (Dietzel et al., 1989).

Modulation of Pump Activity

The activity of the glial cell Na^+/K^+ ATPase increases following reactive gliosis. In rat cortex, pump activity is approximately twice as high in cobalt-induced epileptogenic tissue as it is in control tissue (Onozuka et al., 1987). Similarly, astrocytic pump activity increases ~40% in brain tissue from rats with experimentally induced hepatogenic encephalopathy (Albrecht et al., 1985). The increase in Na^+/K^+ ATPase activity will result in an enhancement of active uptake, and, perhaps to better $[K^+]_o$ regulation in damaged brain tissue.

PASSIVE POTASSIUM UPTAKE

Potassium Accumulation

A large component of K^+ accumulation in glial cells is ouabain-insensitive. This component is due to passive uptake and occurs by one of two mechanisms: (1) by coupled transport, via a $Na^+/K^+/2Cl^-$ or a K/Cl cotransporter, and (2) by K^+ influx accompanied by Cl^- influx through distinct channels and driven by Donnan forces (Boyle and Conway, 1941). Passive K^+ uptake accompanied by Cl^- influx results in an increase in internal osmolarity and to an influx of water into glial cells. Thus, passive uptake is, of necessity, coupled to glial cell swelling and to a decrease in the volume fraction of extracellular space. Indeed, neuronal activity and increased $[K^+]_o$ is often associated with decreases in extracellular volume (Dietzel et al., 1980; Ransom et al., 1985). It is important to note that decreased extracellular volume may be caused by neuronal as well as by glial cell swelling (Dietzel et al., 1989), although the extracellular volume changes seen in CNS white matter are primarily glial in origin (Ransom et al., 1985).

The *in situ* glial cell-olfactory slice experiment cited above (Ballanyi et al., 1987) provides evidence that passive K^+ uptake occurs *in vivo*. Potassium accumulation seen in these cells following electrical stimulation (Figure 47-4) was largely blocked by Ba^{2+} application, as was the stimulus evoked increase in $[Cl^-]_i$, suggesting that K^+ accumulation occurs, in part, by a passive influx of K^+ and Cl^- driven by Donnan forces. The observed 16 mM increase in intracellular $[K^+]$ was too large to be due solely to $K^+ + Cl^-$ uptake, however, suggesting that efflux of some other ion, perhaps Na^+, helped to maintain cellular osmotic balance. A K^+ cotransport mechanism appeared not to play a significant role in K^+ uptake.

Passive K^+ uptake has also been demonstrated in cultured glial cells, with both K^+ cotransport and K^+ flux through channels observed. In cultured astrocytes, a furosemide- and bumetanide-sensitive K^+ cotransporter led to K^+ accumulation following a rise in $[K^+]_0$ (Walz and Hinks, 1985; Walz, 1987). In at least some preparations, transport was dependent on both external Na^+ and Cl^-, suggesting that K^+ was taken up by a $Na^+/K^+/2Cl^-$ cotransporter (Kimelberg and Frangakis, 1985; Tas et al., 1987). Passive $K^+ + Cl^-$ influx through distinct channels was also observed in cultured astrocytes (Walz, 1987) and in cultured mouse oligodendrocytes (Ballanyi and Kettenmann, 1990).

Potassium cotransport into glial cells may be linked to active transport by a transmembrane Na^+ cycle (Walz and Hinks, 1986). Potassium influx via a $Na^+/K^+/2Cl^-$ cotransporter results in an increase in $[Na^+]_i$, which has the dual effect of stimulating the Na^+/K^+ ATPase and providing the excess internal Na^+ needed to actively pump additional K^+ into the cell. Transported Na^+ is recycled in this manner, and K^+ is accumulated within glial cells without disturbing internal Na^+ levels.

The relative contributions of K^+ cotransport and Donnan mechanisms responsible for passive K^+ accumulation in glial cells is unclear. Cotransport plays a minor role in at least one *in situ* preparation (Ballanyi et al., 1987) while its dominates in some astrocyte cultures (Walz, 1987). The issue is highlighted by the dramatic uptake differences seen in primary cultures of mouse and rat astrocytes. In a comparative study (Walz and Kimelberg, 1985), the cotransport inhibitor furosemide blocked K^+ uptake substantially in rat cells but had no effect on mouse cells. In contrast, the K^+ channel blocker Ba^{2+} had a much larger inhibitory effect in mouse cells than it did in rat cells. These differences in two closely related species could arise from a differential expression of K^+ channels and serve as a warning that cultured glial cells may be an inadequate model of K^+ uptake for glial cells *in vivo*.

Modulation of Potassium Uptake

Low Cl^- permeability may prove to be the limiting factor in glial cell K^+ accumulation driven by Donnan forces. In this respect, it is interesting to note that glial cell Cl^- permeability can be increased under certain conditions. Astrocyte Cl^- conductance increases as cells are depolarized (Bevan et al., 1985). Glial cells also possess large-conductance Cl^- channels that are normally inactive but open when the membrane is detached from a cell (in inside-out patches), suggesting that these channels are normally in an inactivated state (Gray et al., 1984; Barres et al., 1988). Channel activation may be triggered by an increase in neuronal activity (Barres et al., 1988). In addition, glial cells possess Cl^--permeable $GABA_A$ receptors (MacVicar et al., 1989; Malchow et al., 1989). Thus, neuronal activity may result in an increase in Cl^- permeability and to enhanced K^+ + Cl^- influx into glial cells.

Large glial cell depolarizations associated with spreading depression and hypoxia may also result in the activation of voltage-gated K^+ channels, such as delayed rectifier, A-type and Ca^{2+}-activated K^+ channels, which are not open near the normal cell resting potential. The resulting increase in glial cell K^+ permeability might lead to an increase in K^+ accumulation as well as to enhanced K^+ spatial buffering.

POTASSIUM SPATIAL BUFFERING

Theory

Excess K^+ released from active neurons can be buffered by its transfer to regions of neural tissue where $[K^+]_o$ is lower. This transfer can occur by diffusion of K^+ through extracellular space or by current flow through glial cells. The latter process is termed "K^+ spatial buffering" (Orkand et al., 1966). Astrocytes, due to their distribution within the central nervous system and their electrical coupling, are believed to be the glial cell principally involved in spatial buffering within the brain.

The K^+ spatial buffering process operates as follows: A local increase in $[K^+]_o$, generated by neuronal activity, results in an inbalance of K^+ equilibrium across glial cell membranes and to an influx of K^+ into glial cells. This K^+ influx depolarizes glial cells and drives out an equal amount of current from other cell regions. This current efflux will also be conducted by K^+ because glial cells are principally permeable to K^+. The net effect of this current flow is to transfer K^+ from regions where $[K^+]_o$ is higher to regions where $[K^+]_o$ is lower. The same amount of K^+ leaves the cells as enters them. Thus, to a first approximation, the spatial buffering mechanism does not result in an accumulation of K^+ within glial cells.

Spatial buffering currents are accompanied by a change in the osmotic balance in extracellular space and within glial cells (Dietzel et al., 1980). Although spatial buffering current is carried exclusively by K^+ across the glial cell membrane, it is carried largely by Na^+ and Cl^- in extracellular space and by Cl^- and organic anions, as well as by K^+, inside glial cells. As a result, in regions where $[K^+]_o$ is raised, the osmolarity decreases substantially in extracellular space and increases slightly within glial cells. (The change in osmolarity is proportional to the fraction of current carried by anions.) These osmotic shifts result in an influx of water into glial cells and to a shrinking of extracellular space (Dietzel et al., 1980).

Electrical Coupling

The distance over which K^+ is transported by the spatial buffering process is not limited by the size of a single astrocyte. Astrocytes are linked together by an extensive array of gap junctions which result in electrical coupling between cells (see Chapter 19). Thus, spatial buffering currents which enter one glial cell can flow through the glial syncytium and exit from a distant cell. In practice, spatial buffering through a glial syncytium is limited by the space constant of the syncytium. Current cannot be efficiently conducted over a long distance within a syncytium which has a short space constant.

The space constant of the astrocyte syncytium within the brain is difficult to assess because such basic cell properties as the specific membrane conductance, the distribution of membrane conductance, and the degree of electrical coupling between cells are not known. Nevertheless, estimates of the space constant of glial syncytia have been made. In the rat cerebellar cortex, a space constant of 200 μm was estimated, based on the transport of K^+ induced by an electric field (Gardner-Medwin, 1983a). Similar techniques yielded a shorter space constant of <60 μm for the frog optic tectum (Gardner-Medwin, 1985). A space constant of 104 μm was estimated for single processes (but not a syncytium) of acutely isolated type-1 rat optic nerve astrocytes (Barres et al., 1990). These space constant estimates suggest that the distance over which the K^+ spatial buffering process is effective is limited to hundreds of micrometers.

It should be noted that the transport of K^+ by spatial buffering can extend beyond the limits of sin-

gle cells, even if these cells are not electrically coupled. In the absence of electrical coupling, excess K^+ could be transported via spatial buffering to the distal extremes of a cell, where it would result in $[K^+]_o$ increases and the generation of spatial buffering currents in other cells with overlapping processes. The efficiency of such a spatial buffering process through "indirectly coupled" glial cells has not been determined.

Spatial Buffering in the Cortex

Several lines of evidence suggest that K^+ spatial buffering plays an important role in regulating activity-dependent K^+ variations within the brain. Studies in the rat cortex and cerebellum demonstrated that substantial K^+ transport occurs through current flow within cells. When a constant current was applied to the cortical surface, the amount of K^+ transported within the tissue was much larger than that predicted assuming that K^+ flux was confined solely to extracellular space (Gardner-Medwin, 1983b; Gardner-Medwin and Nicholson, 1983). The results indicated that five times as much K^+ was transported by current flow within cells as was transported through extracellular space. A large fraction of this cellular current was believed to be conducted through the glial syncytium. The results suggest that spatial buffering currents play an important role in regulating activity-dependent $[K^+]_o$ increases (Gardner-Medwin, 1983a).

Potassium spatial buffering currents have been shown to play an important role in clearing $[K^+]_o$ increases evoked by electrical stimulation in the cat cortex. Current source-density analysis of field potentials showed that current entered cells in the middle cortical layers, where evoked $[K^+]_o$ increases are largest, while currents left cells both above and below this region (Dietzel et al., 1989). These currents served to transfer K^+ away from the primary $[K^+]_o$ increase, removing 3 to 5 mM of K^+ from extracellular space during the 10 seconds of stimulation.

Evidence for K^+ spatial buffering in the cat cortex also comes from measurements of $[K^+]_o$ increases and shrinkage of extracellular space (Dietzel et al., 1980, 1982). During electrical stimulation, $[K^+]_o$ increases, although largest in the middle cortical layers, also occurred in more superficial and deeper cortical layers. Shrinkage of extracellular space, in contrast, was more sharply localized to the middle cortical layers. A potassium spatial buffer current flow, entering glial cells where $[K^+]_o$ increases are greatest, accounts nicely for these observations, as a reduction in extracellular space is predicted to occur in regions of K^+ influx into glial cells, but not in regions of K^+ efflux.

A study in the rat supraoptic nucleus (Coles and Poulain, 1991) also suggests that K^+ spatial buffering plays an important role in $[K^+]_o$ clearance. Antidromic activation of neurons in this preparation produced a $[K^+]_o$ increase only 7% of that predicted assuming no K^+ regulatory mechanisms. Evidence suggested that diffusion and net uptake of K^+ into neurons and glial cells played little role in K^+ regulation. Most of the excess K^+ appeared to be transported by a spatial buffer current to the subarachnoid space. (If K^+ currents were preferentially directed by glial cells to the subarachnoid space, this would constitute an example of K^+ siphoning.)

POTASSIUM SIPHONING

Theory

The spatial buffering mechanism, as originally proposed, presumes that K^+ conductance is distributed uniformly over the surface of glial cells. Influx of K^+ in regions where $[K^+]_o$ is raised is balanced by a K^+ efflux from all other glial cell regions. This current flow results in excess K^+ being transferred somewhat indiscriminately to all neural regions where $[K^+]_o$ is lower.

As reviewed in the following section, however, K^+ conductance in at least some glial cells is not distributed uniformly over the cell surface, but rather is localized preferentially to cell endfeet. As a result of this K^+ conductance localization, spatial buffering currents will be directed to specific regions of the nervous system. Instead of K^+ efflux occurring from all glial cells regions, efflux will be shunted preferentially from glial cell endfeet (Figure 47-5). This spatially directed variant of K^+ spatial buffering is termed "K^+ siphoning" (Newman et al., 1984) and results in, in at least some tissues, a more efficient regulation of $[K^+]_o$.

Within the brain, glial cell endfeet terminate at the pial surface, adjacent to the subarachnoid space, and at the surface of blood vessels. In the retina, the endfeet of Müller cells, the principal glial cells in this tissue, terminate at the inner limiting membrane, adjacent to the vitreous humor. Thus, K^+ siphoning results in the preferential transfer of K^+ to the subarachnoid space and, in the retina, to the vitreous humor. These fluid-filled spaces function as large reservoirs or sinks for the K^+. It is stressed that K^+ siphoning is simply a specialized form of K^+ spatial buffering in which K^+ currents are preferentially di-

FIG. 47-5. Regulation of $[K^+]_o$ by K^+ siphoning in the amphibian retina. Light-evoked neuronal activity generates $[K^+]_o$ increases in the inner plexiform layer (*IPL*) and the outer plexiform layer (*OPL*). These $[K^+]_o$ increases lead to K^+ influx into Müller cells (*M*) in the plexiform layers (*arrows*) and to K^+ efflux from the cell endfoot. The K^+ fluxes establish a radially oriented current within the retina (*solid lines*) which results in the transfer of K^+ from the plexiform layers to the vitreous humor. The current also generates a vitreous positive potential, the electroretinogram b-wave. [Modified from Newman (1985b), with permission.]

rected out of glial cells through high conductance regions.

Potassium siphoning also directs excess K^+ to the surface of blood vessels and results in a rapid $[K^+]_o$ increase at the blood vessel wall following neuronal activity (Paulson and Newman, 1987). The consequence of this $[K^+]_o$ rise is not known, although it has been implicated in the local regulation of blood flow within the brain (Paulson and Newman, 1987). [Brain vessels probably do not function as effective K^+ sinks as the blood-brain barrier is largely impermeable to K^+ and K^+ transport activity in vascular endothelial cells is relatively low (Goldstein, 1979).]

Localization of K^+ conductance to glial cell endfeet has the additional consequence of maintaining the cell membrane potential at a hyperpolarized value when $[K^+]_o$ is raised within the parenchyma. The glial cell membrane potential remains close to the K^+ equilibrium potential at the cell endfoot because a large fraction of the cell conductance resides in the endfoot. When K^+ is raised within nervous tissue, $[K^+]_o$ remains relatively low at the endfoot and the cell membrane potential is raised relatively little. This "clamping" of the cell membrane potential has the consequence of maintaining a large driving force for the influx of K^+ into glial cells and results in enhanced K^+ siphoning currents as well as greater passive K^+ accumulation.

Distribution of Potassium Channels in Glial Cells

Müller Cells. The distribution of K^+ conductance over the surface of glial cells has been best characterized in Müller cells, the principal glial cell of the vertebrate retina (see Chapters 4 and 56). In amphibian Müller cells, input resistance measurements indicate that ~90% of the total cell conductance is localized to the endfoot region (Newman, 1984, 1985a). This localization has been confirmed in voltage-clamp studies which demonstrate that the density of K^+ channels is far higher on the endfoot (38- to 95-fold) than in other cell regions (Brew et al., 1986; Newman, 1993).

Potassium channels are also localized in mammalian Müller cells, although their distribution is more complex. Conductance is high at the vitreal endfoot, as it is in amphibians. In species with vascularized retinas, K^+ conductance is also high in regions near the cell soma (where cell processes contact blood vessels), suggesting that Müller cell "endfeet" contacting blood vessels have high K^+ conductance (Newman, 1987).

Astrocytes. The endfeet of amphibian astrocytes also have high K^+ conductance (Figure 47-6). In freshly isolated salamander cells, specific membrane conductance was approximately tenfold greater at cell endfeet than in other cell regions (Newman, 1986).

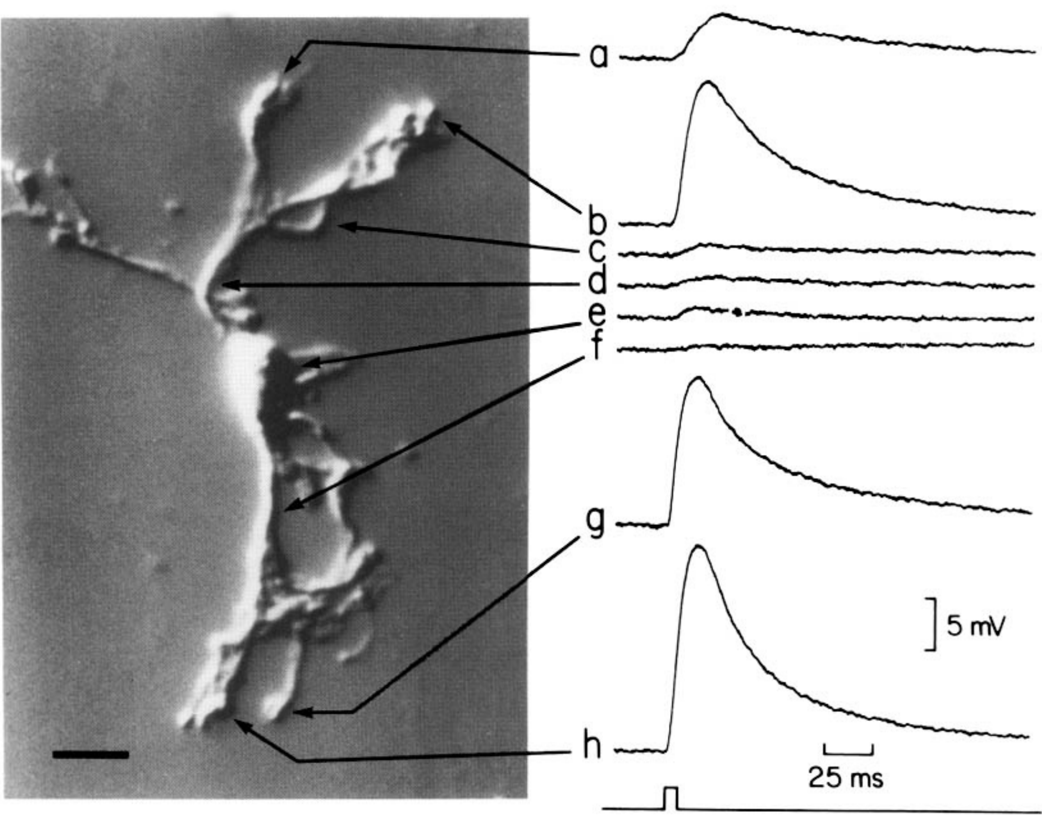

FIG. 47-6. Distribution of K^+ conductance in an amphibian astrocyte from the salamander optic nerve (freshly dissociated cell). Responses, recorded from the soma of the cell shown at left, are displayed at right. Responses were evoked by ejection of a high $[K^+]$ solution onto discrete regions of the cell (*arrows*). Much larger depolarizations were evoked when $[K^+]_o$ was raised at cell endfeet (*a, b, g, h*) than at cell processes (*c, d, f*) or the cell soma (*e*). Results indicate that local cell K^+ conductance is ~10-fold higher at the cell endfeet than in other cell regions. Similar results have been obtained from Müller cells of the amphibian retina. Scale bar = 20 μm. [From Newman (1986), with permission.]

The distribution of K^+ conductance in mammalian astrocytes has not yet been determined.

Potassium Channels

The type or types of K^+ channels which carry spatial buffer currents in glial cells determine, in part, the effectiveness of the K^+ buffering process. As reviewed in Chapter 11, glial cells possess several different types of voltage-gated K^+ channels. Only those channels which have a substantial open probability at the cell resting membrane potential will participate in the spatial buffering process. In mammalian astrocytes, it is not yet known which K^+ channel(s) constitute the resting K^+ conductance.

In amphibian Müller cells, in contrast, voltage-clamp studies have demonstrated that an inward rectifier K^+ channel is, for practical purposes, the only channel open at the normal resting potential (Brew et al., 1986; Newman, 1993). Channel density is low on most cell regions, but is high on the vitreal surface of the endfoot, where the density can exceed 100 active channels per μ^2. The conductance of this channel increases as the membrane potential is hyperpolarized past the K^+ equilibrium potential and as $[K^+]_o$ is raised. The channel is blocked by micromolar concentrations of Ba^{2+} (Newman, 1989).

The effect of the voltage- and K^+-dependent properties of Müller cell inward rectifying K^+ channels has been assessed in simulations of K^+ siphoning in the amphibian retina (Figure 47-7; Newman, 1993). Modeling results indicate that channel properties augment the clearance of K^+ due to K^+ siphoning. Given an instantaneous increase of K^+ from 2.5 to 3.5 mM in the inner plexiform layer, channel properties result in a 23% enhancement in the rate of K^+ clearance from the retina. This enhancement is increased to 137% for an initial K^+ increase to 12 mM.

Potassium Siphoning in the Retina

Potassium siphoning plays an important role in clearing light-evoked K^+ increases from the retina.

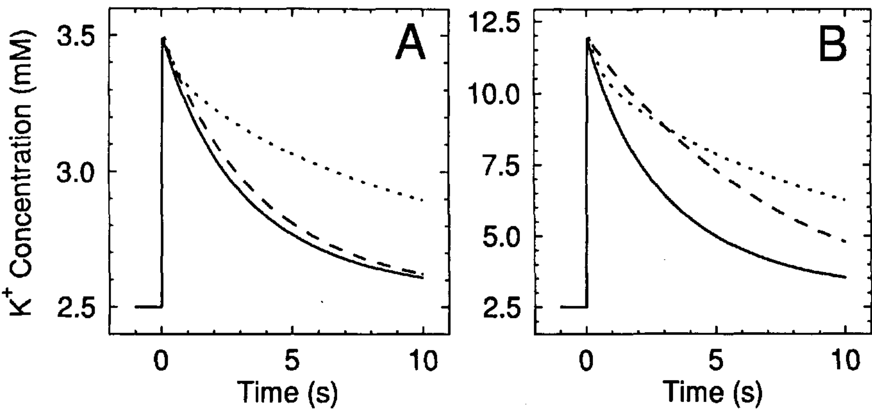

FIG. 47-7. Simulations of K^+ clearance from the amphibian retina. The time course of K^+ removal from the inner plexiform layer is shown following an instantaneous $[K^+]_o$ increase: (A) from 2.5 mM to 3.5 mM or (B) from 2.5 mM to 12 mM. The simulated increase was restricted to the inner plexiform layer. Potassium clearance was modeled for: *1*, K^+ siphoning with inward rectifying Müller cell K^+ channels (solid lines); *2*, K^+ siphoning with Ohmic Müller cell K^+ channels (*dashed lines*); *3*, K^+ diffusion through extracellular space (*dotted lines*). Potassium siphoning was more effective in clearing K^+ from the retina than was K^+ diffusion. Inward rectifying K^+ channels augmented the siphoning process, particularly when $[K^+]_o$ was raised to 12 mM. [From Newman (1993), with permission.]

When the retina is stimulated by light, a $[K^+]$ increase is observed in the vitreous humor. This increase is almost entirely abolished when Müller cell K^+ channels are blocked by Ba^{2+} (Karwoski et al., 1989). The Ba^{2+} blockade of Müller cell siphoning current not only abolishes the transfer of K^+ to the vitreous, it also results in an enlargement in light-evoked $[K^+]_o$ increases within the retina (Figure 47-8). In the toad retina, Ba^{2+} block of siphoning results in light-evoked $[K^+]_o$ increases which are more than double control levels (Oakley et al., 1992), while in the cat, Ba^{2+} block results in K^+ increases that are almost triple control levels (Frishman et al., 1992). In the cat, much of the K^+ is siphoned to the subretinal space (at the photoreceptor layer) rather than to the vitreous, because Müller cell K^+ conductance is largest at the distal end of cells in this species (Newman, 1987). In both the cat (Frishman et al., 1992) and toad (Oakley et al., 1992), this siphoning current helps to buffer the light-evoked $[K^+]_o$ decrease generated by the photoreceptors.

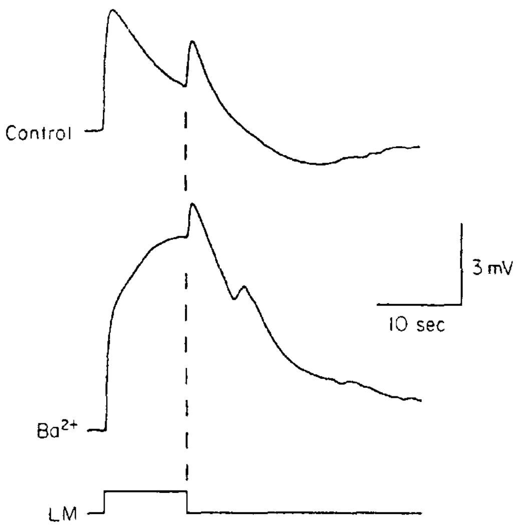

FIG. 47-8. Potassium siphoning in the toad retina (isolated retina preparation). Under control conditions, a 10-second light flash evoked a $[K^+]_o$ increase in the inner plexiform layer, which had both ON and OFF components. Addition of 200 μM Ba^{2+}, which blocked Müller cell K^+ channels, resulted in a substantial increase in the $[K^+]_o$ response. Experiments showed that the $[K^+]_o$ increase was due to the interruption of K^+ siphoning currents to both the vitreous humor and to the subretinal space. The 3 mV $[K^+]_o$ scale bar corresponds to ~0.51 mM. [Modified from Oakley et al. (1992), with permission.]

The effects of Ba^{2+} on K^+ regulation in the retina have also been modeled (Karwoski et al., 1989). Simulations predicted that in the amphibian retina, Ba^{2+} block of Müller cell channels should result in a severe reduction in the light-evoked $[K^+]_o$ increase in the vitreous humor and to an enhancement of the $[K^+]_o$ increase in the inner plexiform layer. The predicted changes matched the experimental results closely.

Theoretical Considerations

The K^+ siphoning process is critically dependent on the distribution of K^+ channels in glial cells. In amphibian Müller cells, for instance, high conductance at the cell endfoot (comprising 90% of the total cell conductance) ensures that most K^+ entering Müller cells from regions of raised $[K^+]_o$ will be directed out

the endfoot and into the vitreous humor. On the other hand, modeling studies indicate that a maximal amount of K^+ can be transferred away from the retina when only 50% of the cell K^+ conductance is localized to the endfoot (Brew and Attwell, 1985). Under these conditions, however, a greater percentage of the excess K^+ would be transferred to other retinal regions instead of the vitreous. Given that ~90% of total cell conductance is localized to the endfoot, it appears that the directed flow of K^+ current away from the retina is of greater importance than is the maximizing of K^+ transferred.

Effective K^+ siphoning presumes that K^+ current flow is not limited by the resistivity of the glial cytoplasm. This condition does not always hold. In the long slender Müller cells of the central portion of the rabbit retina, for instance, the estimated cell space constant is considerably shorter than the cell length, resulting in a substantial attenuation of siphoning currents (Eberhardt and Reichenbach, 1987).

Generation of Field Potentials: An Epi-Phenomenon of K^+ Spatial Buffering?

Electroretinogram. Light-evoked $[K^+]_o$ increases in the inner and outer plexiform layers of the retina establish a K^+ siphoning current within Müller cells directed from the retina to the vitreous humor. This intracellular current is balanced by a return current flow through extracellular space, directed from the vitreous to the distal retina (Figure 47-5). This current generates a vitreal positive potential across the retina which is believed to be the origin of the b-wave of the electroretinogram (ERG) (Newman, 1980). Similarly, other light-evoked $[K^+]_o$ variations in the retina are thought to evoke K^+ siphoning currents in Müller cells, leading to the generation of other ERG components, including the slow PIII potential (Witkovsky et al., 1975), the M-wave (Karwoski and Proenza, 1977; Sieving et al., 1986a), and the scotopic threshold response (Sieving et al., 1986b). Thus, the generation of these retinal field potentials may be thought of as epi-phenomena arising from the regulation of $[K^+]_o$ by Müller cell K^+ siphoning.

Electroencephalogram. Components of the electroencephalogram (EEG) may also be generated by K^+ siphoning or K^+ spatial buffering currents (Cohen, 1970). Fast components of the EEG occur more rapidly than observed activity-dependent variations in $[K^+]_o$ within the brain. Thus, these components must be generated directly by neurons. Slow EEG components, on the other hand, are sometimes correlated with variations in glial cell membrane potential and $[K^+]_o$ and could arise from current flow through astrocytes (Castellucci and Goldring, 1970; Somjen, 1970; Heinemann et al., 1979; see Chapter 20).

RELATIVE EFFECTIVENESS OF K^+ CLEARANCE MECHANISMS

Glial Clearance Mechanisms Operate Simultaneously

The K^+ regulatory mechanisms outlined above operate simultaneously to clear $[K^+]_o$ increases from extracellular space. It is important to note, however, that the mechanisms do not act independently and that operation of one mechanism may influence the operation of others.

There is a tradeoff between K^+ transfer and K^+ accumulation processes. Both K^+ spatial buffering and siphoning mechanisms depend on the selective permeability of glial cells to K^+. These mechanisms become less efficient as cell permeability to other ions, such as Cl^-, is increased. For cells with a large Cl^- permeability, much of the inward K^+ spatial buffering current generated by a $[K^+]_o$ increase will be balanced by a local influx of Cl^-. Thus, less K^+ will be transported to a distant site by K^+ efflux from other cell regions.

Spatial buffer current can also affect the efficiency of passive uptake mechanisms. Spatial buffer currents within glial cells are carried, in part, by anions as well as by K^+. Thus, both K^+ and Cl^- accumulate within glial cells in regions of K^+ influx (Dietzel et al., 1980). The increases in $[K^+]_i$ and $[Cl^-]_i$, in turn, reduce the magnitude of K^+ and Cl^- influx due to cotransport and Donnan mechanisms.

One of the most important questions in the field of K^+ regulation concerns the relative contributions of the different regulatory mechanisms. We are still a long way from answering this question definitively. As discussed in the following sections, however, theoretical as well as experimental studies have provided some general rules that indicate the relative importance of different regulatory mechanisms under specific conditions.

Temporal Characteristics

The temporal characteristics of activity-dependent $[K^+]_o$ variations play an important role in determining which mechanisms are effective in clearing K^+ from extracellular space. Active uptake of K^+ into neurons occurs rather slowly, having a time constant of tens of seconds (Gardner-Medwin, 1983a). Thus, active neuronal uptake is relatively ineffective in buf-

fering $[K^+]_o$ variations. Passive K^+ uptake will be effective in clearing brief $[K^+]_o$ increases, but will be less effective in clearing more prolonged increases as the capacity of glial cells (and neurons) to accumulate K^+ becomes saturated. Diffusion and spatial buffering mechanisms, in contrast, retain their ability to transfer K^+ to a distant site, even when a $[K^+]_o$ increase is prolonged (Gardner-Medwin, 1986).

Spatial Characteristics

The spatial characteristics of $[K^+]_o$ variations also play an important role in determining which $[K^+]_o$ clearance mechanisms dominate. Both active and passive uptake processes operate independently of the spatial extent of a $[K^+]_o$ increase. Uptake rates will be the same whether the size of a $[K^+]_o$ increase is large or small. Diffusion and K^+ spatial buffering, in contrast, are critically dependent on the spatial extent of an increase. For restricted $[K^+]_o$ increases (less than a few hundred micrometers), diffusion is more effective than is spatial buffering in dispersing K^+ (Gardner-Medwin, 1986). As the size of a $[K^+]_o$ increase grows, spatial buffering begins to dominate. In the rat cortex, the crossover point, when spatial buffering and diffusion are equally effective, has been estimated to occur for a sinusoidally modulated $[K^+]_o$ increase with a wavelength of ~700 μm (Gardner-Medwin, 1986). As a $[K^+]_o$ increase grows still larger, spatial buffering will be limited by the space constant of the glial syncytium. In the extreme case, when $[K^+]_o$ is raised uniformly throughout a tissue, both diffusion and spatial buffering will be completely ineffective in reducing the $[K^+]_o$ increase.

The K^+ siphoning process provides an exception to this rule. Even when $[K^+]_o$ is increased uniformly within the brain parenchyma, $[K^+]$ in the subarachnoid space at the pial surface of the brain, will remain relatively low. Thus, astrocyte endfeet at the pial surface will experience a $[K^+]$ that is lower than that within the brain and a K^+ siphoning current will be generated. This current will transfer K^+ from the brain to the cerebral spinal fluid in the subarachnoid space.

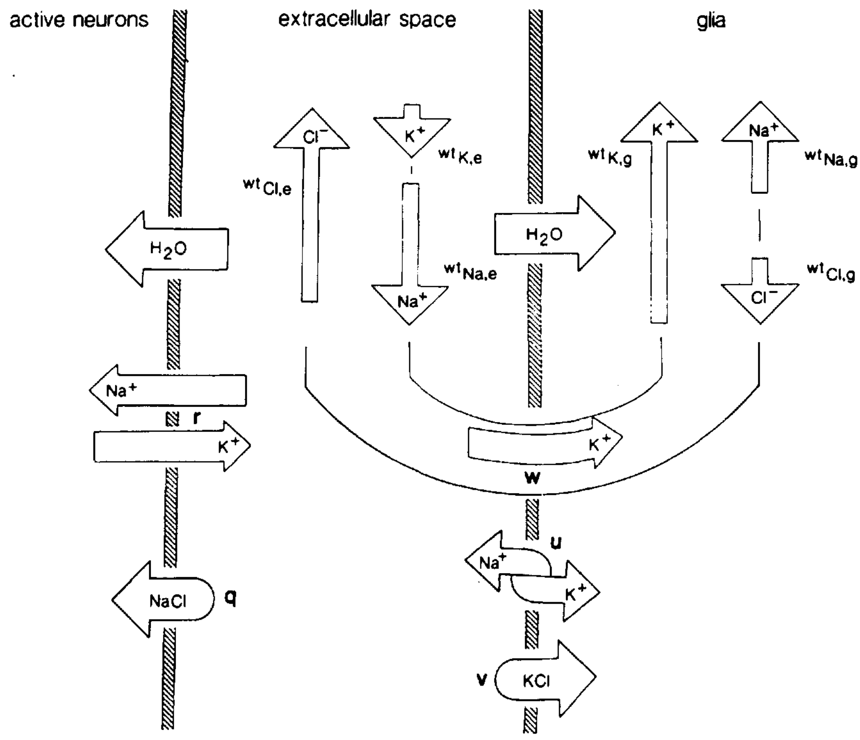

FIG. 47-9. Model of K^+ regulation in the cat cerebral cortex. The diagram indicates ion fluxes across the membrane of an active neuron (*left*), across the membrane and through a glial cell (*right*), and through extracellular space (*center*). In the model, neuronal activation leads to an influx of Na^+ and an efflux of K^+ from the neuron (*r*), which results in an increase in $[K^+]_o$. The $[K^+]_o$ increase, in turn, leads to passive uptake of KCl (*v*) and to active K^+ uptake (*u*) into the glial cell. A K^+ spatial buffer current is generated and is carried by K^+ across the glial cell membrane (*w*) and by Na^+, K^+, and Cl^- in extracellular space and within the glial cell. The ion fluxes result in a drop in osmotic pressure in extracellular space and in a rise of osmotic pressure in the neuron and glial cell, driving water into both cells. Calculations give an estimate of the relative contributions of glial regulatory mechanisms to the removal of K^+ from extracellular space: spatial buffering, 45%; active uptake, 39%; passive uptake, 16%. [From Dietzel et al. (1989), with permission.]

Potassium siphoning is most effective when a $[K^+]_0$ increase occurs near a large fluid body. In the retina, this condition is satisfied as all retinal regions lie within 250 μm of the vitreous humor (but see Eberhardt and Reichenbach, 1987). In the brain, K^+ siphoning will be effective for $[K^+]_o$ increases which occur near the cortical surface (perhaps 200 to 1000 μm beneath the surface, depending on the space constant of the glial syncytium). For deeper neural regions, other clearance mechanisms will dominate.

Models of K^+ Regulation

Mammalian Cortex. A comprehensive model of K^+ regulation in the cat cortex has been used to estimate the relative contributions of different regulatory mechanisms (Dietzel et al., 1989) (Figure 47-9). The model analyzes K^+ uptake during a 10-second period of stimulation by incorporating experimentally measured variations in intracellular and extracellular $[K^+]$, $[Na^+]$, and $[Cl^-]$ as well as changes in the extracellular volume fraction. Results indicate that during the stimulation period, the equivalent of 3 to 5 mM K^+ is removed from extracellular space by spatial buffering, 3.4 mM by action of a glial Na^+/K^+ ATPase, and 1.4 mM by passive K^+ uptake into glial cells. These estimates are, of necessity, imprecise as all of the parameters affecting K^+ regulation have not been determined in a single preparation.

Amphibian Retina. Potassium clearance has also been analyzed, both experimentally and analytically, using the amphibian retina preparation. As noted above, K^+ siphoning in the retina is selectively blocked by application of micromolar Ba^{2+}. With siphoning blocked, light-evoked $[K^+]_o$ increases within the retina grow larger while the $[K^+]$ increase in the vitreous humor is almost completely abolished. Simulations demonstrate that the small remaining increase in the vitreous (8 to 11% of control) is due to the transfer of K^+ by diffusion through extracellular space (Karwoski et al., 1989). These results demonstrate that K^+ siphoning plays a much more important role in clearing K^+ from the retina than does diffusion.

CONCLUSION

Glial cells possess a number of physiological mechanisms which contribute to K^+ regulation in the central nervous system. These processes, which include active and passive accumulation of K^+ and K^+ transfer by spatial buffer currents, play a dominant role in K^+ homeostasis within the nervous system. These processes have been characterized in both *in situ* and *in vitro* preparations. The relative contribution of each of these processes remains unknown, although general principals regarding their efficacy have been established. One of the challenges for future research is to determine the role that each of these K^+ regulatory mechanisms play in different tissues and for different levels of neuronal activity.

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48 The role of glia in the inactivation of neurotransmitters

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In 1907 Ernesto Lugaro suggested that glial cells might be involved in the inactivation of substances released by neurons, a speculation of remarkable foresight given the primitive knowledge of glial cells and communication between neurons at that time. In the years preceding Lugaro's article, the long-standing controversy between the neuronal and reticular theories of connectivity in the nervous system was being resolved in favor of the neuron theory (Ramón y Cajal, 1989), and the need to explain the propagation of nerve impulses between the separate neurons had become an important problem. According to Bacq (1975), most physiologists thought that the impulse was transmitted from one neuron to another by electrical currents. Although some thought the impulse could be transmitted chemically, there was no evidence that neurons actually secreted active substances until Loewi reported his discovery of *Vagusstoff* in 1921, 14 years after Lugaro's article. The electrical theory of synaptic transmission did not die easily. Despite the accumulating evidence for chemical synaptic transmission over the succeeding years, some physiologists continued to support the electrical mechanism, and it was not until J. C. Eccles converted to the chemical side in 1945 that serious opposition to chemical synaptic transmission ended (Bacq, 1975).

In 1950 George Koelle reported that nonspecific cholinesterase was localized in the nuclei of glial cells in autonomic ganglia, thus providing evidence that glia have the *capacity* to degrade a neurotransmitter. In discussing his findings, Koelle cited the views of de Castro, who regarded synapses as composed of three elements: the pre- and postsynaptic neural elements and the glia in which the terminals are imbedded. Koelle also thought that nonspecific cholinesterase in glia might have two functions—a transmission-terminating capacity and a protective capacity to prevent transmitter overflow from one synapse from inappropriately activating another synapse. Koelle subsequently refined his histochemical technique and determined that the nuclear location of nonspecific cholinesterase was artifactual, but that the glial location was genuine (Koelle, 1951, 1954). He also established that glia in the central nervous system possess nonspecific cholinesterase, but he was unwilling to extend his ideas about the functions of glia to the central nervous system, saying that "the occurrence of nonspecific ChE in nonnervous cells of the rat brain militates against the likelihood of its having an important role in central transmission" (Koelle, 1954). This cautious outlook stands in contrast to his previous, more expansive ideas about the relationship of glia to synaptic function in autonomic ganglia, where he seemingly felt that the relationship of the glial cholinesterase to cholinergic synapses was more clearly defined.

Because acetylcholine and the biogenic amines were the only serious transmitter candidates at that time, evidence that glia have the capability to inactivate other transmitters was not developed until later. In 1970 Hökfelt and Ljungdahl studied the uptake of [^{3}H]GABA by autoradiography. Although they clearly considered various types of glia as potentially responsible for some of the uptake, the resolution of their method was insufficient to firmly establish the cellular elements responsible. Henn and Hamberger (1971) compared the uptake of γ-aminobutyric acid (GABA), norepinephrine, dopamine, and serotonin by a cell fraction enriched in glial cells with that of a cell fraction enriched in neuronal perikarya, and argued that glia are capable of removing transmitters that overflow from the synaptic cleft. This work was part of a burgeoning interest in GABAergic function and was followed rapidly by a substantial number of studies on the abilities of glia to take up and metabolize transmitters (reviewed by Iversen and Kelly, 1975).

As we discuss in this chapter, glial cells, and astrocytes in particular, envelop synapses in a manner that would enable them to intercept transmitter molecules that overflow from the synaptic cleft, and they are equipped with the transport systems and enzymes that are necessary to degrade most known neurotransmitters. Thus the idea that astrocytes are appropriately placed and equipped to inactivate and

degrade transmitters is supported by good evidence. What is missing is a firm estimate of the quantitative importance of the inactivation of transmitters by astrocytes *in vivo* and *direct* evidence that astrocytes might have any regulatory effects on extracellular levels of synaptic transmitters.

THE MICROSCOPIC ANATOMY OF ASTROCYTES LENDS ITSELF TO THE INACTIVATION OF TRANSMITTERS

The structure of a central nervous system synapse and the location of the surrounding astrocyte is illustrated in Figure 48-1, an electron micrograph of a nerve ending in the suprachiasmatic nucleus (van den Pol et al., 1992). As indicated by the large vertical arrows, astrocytic processes closely surround the synaptic terminal. Vesicular release of the synaptic transmitter, glutamate in this case, is thought to occur at a specific release zone located at the synaptic thickening where the transmitter can immediately interact with its receptor. The mechanisms that inactivate transmitters differ among the various transmitter compounds but include degradation by extracellular enzymes, recapture by the neuron via specific transport systems, diffusion from the synaptic cleft, and inactivation by astrocytes. To reach the astrocyte, however, a transmitter molecule must not only escape from the cleft (in the region of the synaptic thickening) but must also escape from reuptake or enzymatic degradation as it diffuses through the interneuronal space surrounding the cleft. Thus, the amount of transmitter that reaches the astrocyte depends on the efficiency of the neuron's inactivating mechanisms.

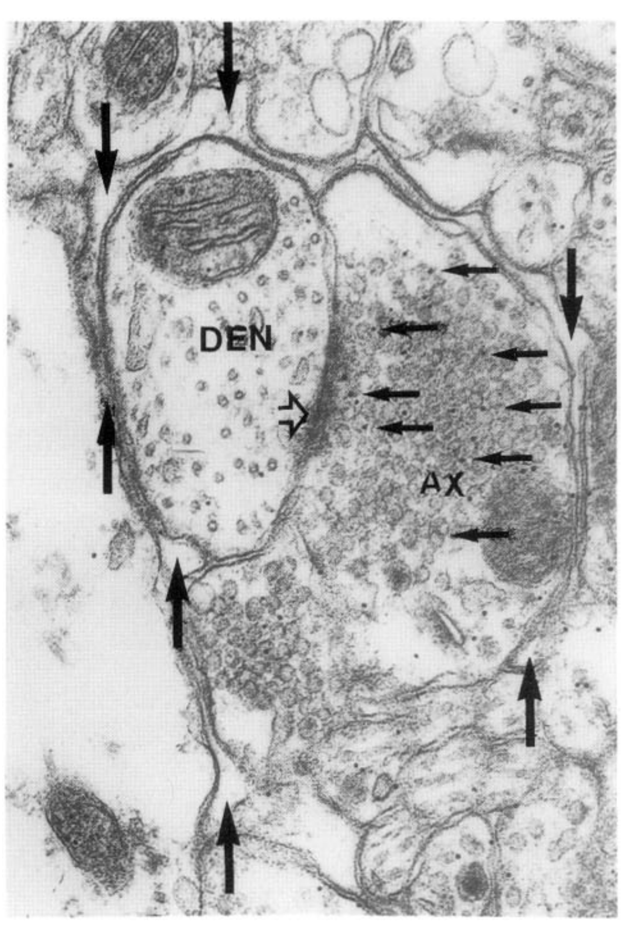
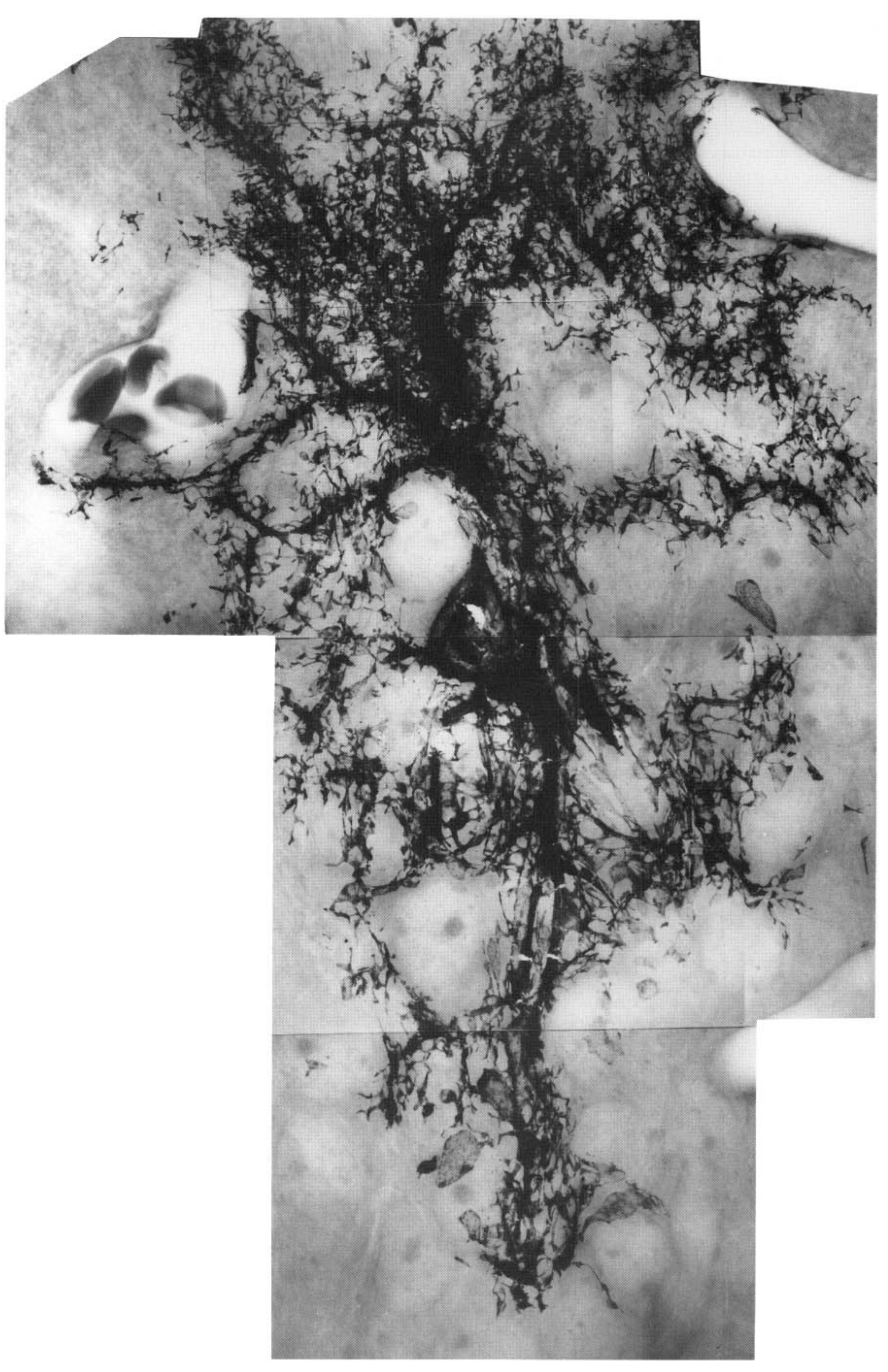


FIG. 48-1. Astrocytic processes surround a glutamatergic terminal in the suprachiasmatic nucleus of the rat and insulate it from the surrounding neuropil. The glutamatergic terminal (*AX*) is immunochemically labeled with colloidal gold particles (*small horizontal arrows*). It synapses (*open arrow*) with a dendrite (*DEN*). Astrocytic processes are indicated by *large vertical arrows*. [From van den Pol et al. (1992), with permission.]

Although astrocytic cell bodies appear small in diameter when visualized with ordinary histochemical stains, astrocytes themselves are large in the sense that they have many exceedingly fine processes that penetrate a large volume of tissue and come into contact with many neurons at many locations, including both cell bodies and dendrites. Among the most dramatic demonstrations of these facts are the elegant images made by Kosaka and Hama (1986). Such images (Figures 48-2 and 48-3) clearly show the astrocyte's many fine veillike and sheetlike processes and also show that each astrocyte surrounds many neurons. Since each central nervous system neuron participates in many types of synaptic contacts, each astrocyte is probably equipped to deal with numerous transmitters. At present the number of different transmitters that an astrocyte can inactivate is not known; to my knowledge, there have been no studies of the colocalization of inactivating systems for different transmitters in astrocytes. As is discussed below, inactivating enzymes, such as glutamine synthetase, are present in perisynaptic astrocytic processes. However, the possibility that an astrocytic process is specialized to deal with the specific type of synapse that it surrounds remains a difficult problem.

GLIA POSSESS INACTIVATION SYSTEMS FOR MANY TRANSMITTERS

Table 48-1 provides a list of inactivating systems for neurotransmitters that are known to be present in glial cells. Some neurotransmitters and neuroactive compounds, particularly acetylcholine, adenosine triphosphate (ATP), and peptides, are degraded by ectoenzymes that are present on the external surface



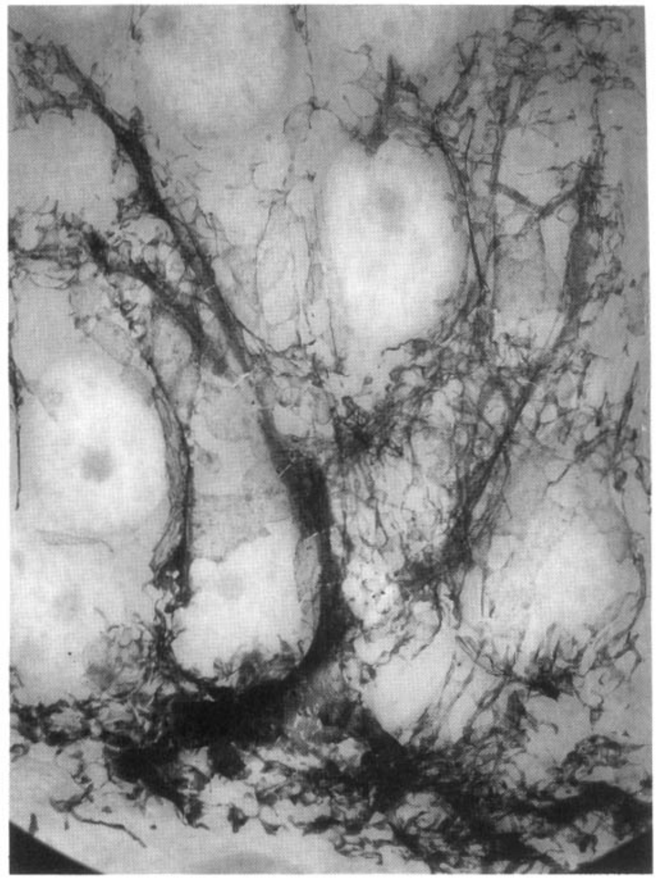
of cells. Other compounds are inactivated by transport systems that remove them from the extracellular space. In such cases, however, uptake is merely the first step of inactivation, as the compounds must be metabolized internally to complete the inactivation process.

Acetylcholine

Evidence that astrocytes contain cholinesterase was presented in the introduction to this chapter. Although glial cholinesterase is the so-called nonspecific type, it is able to degrade acetylcholine very well. In the central nervous system, specific acetylcholinesterase is found only in cholinergic neurons and, in fact, is used as a histochemical marker for those cells.

Excitatory Amino Acids

Astrocytes are thought to play a major role in the inactivation and metabolism of the excitatory amino acid glutamate; they can take up glutamate that escapes from neuronal reuptake systems and metabolize it in the tricarboxylic acid (TCA) cycle or convert it to glutamine. Glutamine produced by



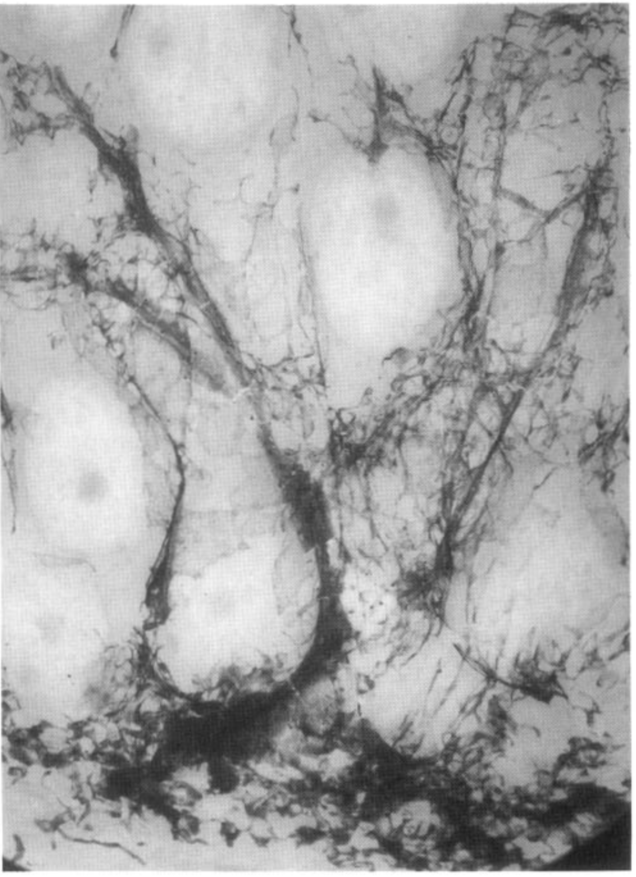


FIG. 48-3. Stereo pair of high-voltage electron micrographs of part of an astrocyte in the granule cell layer of the dentate gyrus. The astrocyte's soma is at the bottom, just left of center. Notice the numerous veillike and leafletlike processes. This astrocyte contacts several unimpregnated neurons in this plane, which can be recognized by the faint images of their nuclei and nucleoli. [Image is from Kosaka and Hama (1986), with permission. Original prints were kindly provided by the authors.]

FIG. 48-2. Photomontage of high-voltage electron micrographs of a Golgi-impregnated astrocyte at the border of the granule cell layer and the molecular layer of the dentate gyrus. 5-μm thick section. Astrocytic processes also extend above and below the plane of this image. The astrocyte's soma is in the center. Note the many fine, sheetlike processes which contact several neurons in this image. Faint images of the nuclei and nucleoli of unimpregnated neurons are evident in the unstained spaces surrounded by the astrocytic processes. A blood vessel with four erythrocytes is at upper left. Scale bar = 5 μm. [Image is from Kosaka and Hama (1986) with permission. Original prints were kindly provided by the authors.]

TABLE 48–1. *Transmitter Inactivating Systems Present in Glial Cells*

Transmitter	Transport systems	Enzymes
Acetylcholine	None	Nonspecific cholinesterase (Koelle, 1950; Koelle, 1954)
Adenosine	Present (Hertz, 1978; Matz and Hertz, 1990)	Adenosine deaminase; adenosine kinase (Matz and Hertz, 1989; Matz and Hertz, 1990)
ATP	None known	EctoATPase (Trams and Lauter, 1978)
Aspartate	Present[a]	Glutamic oxaloacetic transaminase (Schousboe et al., 1977; Schousboe and Hertz, 1984)
Dopamine	Present (Pelton et al., 1981; Kimelberg and Katz, 1986)	Monoamine oxidase (Levitt et al., 1982; Yu and Hertz, 1982; Hansson, 1984; Westlund et al., 1985); catechol-O-methyl transferase (Kaplan et al., 1979; Yu and Hertz, 1982; Hansson, 1984); phenol sulfotransferase (Yu and Walz, 1985)
GABA	Present[a]	GABA transaminase (Schousboe et al., 1977); succinic semialdehyde dehydrogenase
Glutamate	Present[a]	Glutamine synthetase (Martinez-Hernandez et al., 1977; Juurlink, 1982); glutamate dehydrogenase (Aoki et al., 1987; Madl et al., 1988); various transaminases
Glycine	Present[a]	Glycine cleavage system (Sato et al., 1991)
Histamine	Present (Rafalowska et al., 1987; Huszti et al., 1990)	Histamine-*N*-methyltransferase (Rafalowska et al., 1987; Huszti et al., 1990)
N-Acetylaspartyl glutamate (NAAG)	Transporter present for *N*-acetylaspartate and NAAG (O, Saab, personal communication)	
Norepinephrine	Present (Pelton et al., 1981; Kimelberg and Pelton, 1983)	See dopamine
Serotonin	Present (Ritchie et al., 1981; Liesi et al., 1981; Kimelberg and Katz, 1985; Kimelberg and Katz, 1986)	See dopamine

[a]Described by Schousboe in Chapter 15, this volume.

astrocytes is released into the extracellular space, and can be taken up by neurons where it serves as a precursor of glutamate synthesis (Balazs et al., 1970; Berl and Clarke, 1983). This glutamate-glutamine cycle has been studied extensively, and there is ample evidence that glial cells are capable of their part of the cycle (Schousboe, 1981; Waniewski and Martin, 1986; Erecińska and Silver, 1990). The glutamate-inactivating ability of astrocytes also is thought to be important in limiting the excitotoxic effects of glutamate on neurons. The resistance of astrocytes to glutamate-induced cell death undoubtedly helps them to play a protective role. Cultured oligodendrocytes, in contrast, appear to be as vulnerable to glutamate-induced cell death as cultured neurons (Oka et al., 1993). It is interesting that glutamate does not appear to damage cultured oligodendrocytes by a receptor-mediated mechanism but makes the cells vulnerable to free-radical attack by depleting them of cystine and lowering their glutathione content.

Astrocytes possess multiple uptake systems for glutamate (for detailed descriptions see Chapter 15, this volume; Erecińska and Silver, 1990; Flott and Seifert, 1991). One of the transport systems for glutamate is Na^+-dependent and capable of active transport, and a second is Cl^--dependent. Ca^{2+} also stimulates uptake (Flott and Seifert, 1991). Cl^- dependent uptake appears to account for a small fraction of total uptake in primary cultures of astrocytes (Flott and Seifert, 1991), but it is a more important contributor in glioma cells (Waniewski and Martin, 1984). The K_m for Na^+-dependent glutamate transport by cultured astrocytes is comparable to that of neurons, and the V_{max} is somewhat greater in astrocytes than in neurons, indicating that the astrocytes have ample transport capacity to take up glutamate that overflows from the synapse and to participate

in the glutamate-glutamine cycle (Drejer et al., 1982; Schousboe and Hertz, 1984). Because of the complex structural relationship of the synapse and the surrounding astrocytes (see Figure 48-1), the neuronal transport systems are exposed sooner to higher concentrations of synaptically released glutamate. Thus, the higher V_{max} in astrocytes does not indicate that astrocytes take up most of the glutamate released at the synapse.

A Na^+-K^+-coupled glutamate transporter that is located in glial cells was purified recently from rat brain (Danbolt et al., 1992), and glutamate transporters also have been cloned (Pines et al., 1992; Kanai and Hediger, 1992; Storck et al., 1992). Two of these transporters appear to be expressed by astrocytes but differ in sequence (Pines et al., 1992; Storck et al., 1992), indicating that astrocytes possess at least two structurally distinct glutamate transporters. In immunocytochemical studies, the fine processes of astrocytes were particularly strongly stained by antibodies against a glutamate transporter (Danbolt et al., 1992).

Glutamine synthetase is found in both astrocytes and oligodendrocytes but does not appear to be present in neurons in significant amounts (Martinez-Hernandez et al., 1977; Cammer, 1990). Juurlink and Hertz (1991) found that the specific activity of glutamine synthetase was sevenfold higher in cultures of type-2 astrocytes than in cultures of type-1 astrocytes, but the significance of the higher levels in cultured type-2 astrocytes is unclear. Immunocytochemical studies clearly show that glutamine synthetase is present in glial processes closely surrounding glutamatergic synapses (Derouiche and Frotscher, 1991). Glutamine and glutamine synthetase are not associated solely with glutamate and glutamatergic transmission, as glutamine is a major precursor of GABA synthesis (Erecińska and Silver, 1990, Battaglioli and Martin, 1991, Martin and Rimvall, 1993).

Immunocytochemical studies have shown that astroglia *in vivo* exhibit an intense but heterogeneous labeling for glutamate dehydrogenase (GDH), a mitochondrial enzyme that can convert glutamate to the TCA cycle intermediate α-ketoglutarate (Aoki et al., 1987; see Chapter 33, this volume). In contrast, neuronal labeling was uniform but of lower intensity. Intense astrocytic labeling for GDH appears to be associated with two factors. The first is glutamatergic transmission, as high levels of astrocytic GDH are found in regions that have large numbers of glutamatergic terminals. The second is a high level of neuronal activity, as a high level of GDH immunoreactivity in astrocytes is found in regions that are thought to have fewer glutamatergic terminals but stain strongly for cytochrome oxidase, another mitochondrial enzyme that is thought to be associated with high levels of neuronal activity.

Astrocytes also possess other enzymes of glutamate metabolism, including glutamic-oxaloacetic transaminase (GOT, aspartate aminotransferase) and phosphate-activated glutaminase (Schousboe et al., 1977; Schousboe and Hertz, 1984).

The proportion of transmitter glutamate that passes through astrocytes and returns to neurons via the glutamate-glutamine cycle is unclear because (1) the fraction of transmitter glutamate that is recaptured by neurons and (2) the fraction of glutamate that astrocytes convert to glutamine and return to neurons are unknown. Cultured astrocytes convert a major fraction of exogenous glutamate to glutamine and release it to the medium, while some glutamate carbon clearly enters the TCA cycle (Waniewski and Martin, 1986; Farinelli and Nicklas, 1992). The relative amounts of glutamate that are converted to glutamine and enter the TCA cycle differ among studies. Waniewski and Martin (1986) and Farinelli and Nicklas (1992) found that much more glutamate was converted to glutamine than entered the TCA cycle, whereas Sonnewald et al. (1993) reported that about 30% of labeled glutamate was converted to glutamine and that 70% entered the TCA cycle. It is important to note that there are many differences among these experiments, including the sources of astrocytes, concentrations of glutamate, and other incubation conditions. The exact partitioning of the metabolism of glutamate among the various pathways in cultured astroglial cells undoubtedly depends on the availability of other metabolites and precursors, such as ammonia (Waniewski, 1992), thus making extrapolation from *in vitro* systems to intact tissue tenuous at best.

The route by which glutamate enters the TCA cycle also appears to differ among experiments. Yu et al. (1982) reported that glutamate dehydrogenase appears to be responsible for producing most of the α-ketoglutarate that enters the TCA cycle, since the production of $^{14}CO_2$ from [1-^{14}C]glutamate was almost unaffected when GOT, and presumably other transaminases, were completely inhibited with aminooxyacetate. In contrast, Farinelli and Nicklas (1992) concluded that glutamate enters the TCA cycle mainly through transaminases, as the decarboxylation of [1-^{14}C]glutamate was inhibited by 70 to 90% when GOT was inhibited with aminooxyacetate.

The glutamate transporter also takes up L- and D-aspartate (Schousboe et al., 1977; Drejer et al., 1982; Barbour et al., 1991; Flott and Seifert, 1991). L-Aspartate appears to be taken up by astrocytes via the Na^+-dependent transport system at about half the rate of L-glutamate, but does not appear to be

transported at significant rates by the Cl^- or Ca^{2+}-dependent systems (Flott and Seifert, 1991). In contrast to glutamate, which can be metabolized by many enzymes, aspartate is principally converted to oxaloacetate by GOT, and thus the carbon skeleton enters the TCA cycle.

Gamma-Aminobutyric Acid

High-affinity GABA transport has been demonstrated in a variety of kinds of glia in several tissues, including glia-enriched cell fractions (Henn and Hamberger, 1971), cultured astrocytes from brain (Schousboe et al., 1977), satellite glia and Schwann cells in the superior cervical ganglion, satellite glia in dorsal root ganglion, pinealocytes, and gliocytes of the pituitary (for review see Iversen and Kelly, 1975). Although the ability of glia to transport GABA is commonly assumed to be related to GABAergic transmission, the function of a GABA transport system in the dorsal root ganglion is unclear, as these ganglia have no synapses.

Several distinct transport systems for GABA are present in brain. Glia contain both high- and low-affinity transport systems for GABA, but much of the low-affinity uptake is attributable to the high-affinity transport system for taurine (Martin and Shain, 1979). Inhibition studies indicate that the substrate specificities of the high-affinity transport systems differ among glia and neurons. In particular, glial transport is much more strongly inhibited by β-alanine than is neuronal transport, while neuronal transport is more strongly inhibited by 2,4-diaminobutyric acid (DABA) and *cis*-1,3-aminocyclohexane carboxylic acid (ACHC). There are exceptions to this rule, however, as in the rat retina where GABA uptake by Müller cells is more strongly inhibited by 2,4-diaminobutyric acid than by β-alanine (Iversen and Kelly, 1975). In addition, cultured type-2 astrocytes, which exhibit intense GABA uptake, appear to express the neuronal type of GABA transporter, as their GABA uptake system is strongly inhibited by ACHC but not by β-alanine (Wilkin et al., 1983; Johnstone et al., 1986). The presence of a GABA transporter in astrocytes also has been demonstrated by immunocytochemistry with an antibody raised against a GABA transporter isolated from rat brain (Radian et al., 1990). Both glial cells and neurons are stained, and the relative intensity of the glial and neuronal staining varies among brain regions. It is notable that the transporter was found in glial processes but not cell bodies. Multiple molecular forms of the GABA transporter have been cloned from brain recently (Guastella et al., 1990; Borden et al., 1992). The availability of molecular biological tools will doubtless greatly clarify which GABA transport systems are expressed in glia and where the glial transporters are located relative to GABAergic neurons.

High-affinity GABA transport activity in primary cultures of astrocytes from brain is quite variable, but, in general, astrocytes take up GABA less rapidly than glutamate. Some astrocyte cultures have little or no GABA transport capacity, even though they strongly express other astrocytic markers such as GFAP and glutamine synthetase (Levi et al., 1986; R. A. Waniewski, personal communication). One factor that may contribute to this variability is the cellular composition of the cultures. Cultured type-2 astrocytes exhibit markedly stronger uptake of GABA than do type-1 astrocytes (Levi et al., 1986; Johnstone et al., 1986), and the proportion of the two cell types in the cultures depends on culture conditions and the age of the cultures. A second factor may be the presence or absence of regulatory agents, as GABA transport activity responds to neuronally released factors. The ability of astrocytes to take up GABA is increased by prior exposure to β-adrenergic agonists (Hansson and Ronnback, 1991), and a protein released from cerebellar granule cells (Nissen et al., 1992).

Astrocytes in primary culture also take up GABA more slowly than do cultures of cortical neurons, which are mostly GABAergic (Schousboe and Hertz, 1984). This observation contrasts with glutamate uptake which is more rapid in astrocytes than in neurons, and has led to the idea that inactivation of GABA by astrocytes is quantitatively less important than inactivation of glutamate. This suggestion may be premature, however, as it is difficult to extrapolate from cultures to the *in vivo* situation, and the expression of GABA transporters by cultured astrocytes may be regulated by neuronal factors, as noted above.

Under most circumstances glia contain very little GABA and rapidly metabolize labeled exogenous GABA. GABA is degraded in two steps. First, GABA transaminase converts the carbon skeleton of GABA to succinic semialdehyde, which is then converted to the TCA cycle intermediate succinate by succinic semialdehyde dehydrogenase. When $[^3H]$GABA is used as a transport substrate, the label accompanies the carbon skeleton until it enters the TCA cycle when half of the label is lost as ^3H_2O in the first passage of the carbon skeleton through the cycle. Because glia contain little endogenous GABA there is little isotopic dilution when labeled GABA enters the cell, and much of the label is rapidly lost. As a result, GABA uptake by glia is usually enhanced sub-

stantially when GABA transaminase is inhibited (Schon and Kelly, 1974; Iversen and Kelly, 1975). In immunocytochemical studies, GABA-like immunoreactivity is rarely observed in glia unless the animal or tissue is pretreated with a GABA-transaminase inhibitor (Storm-Mathisen and Ottersen, 1986; Palmi et al., 1991). It is notable, however, that O-2A progenitors and oligodendrocytes in rat optic nerve contain GABA (Barres et al., 1990), and that GABA has been found in astrocytes in brainstem (Blomqvist and Broman, 1988).

Monoamines

In addition to studying GABA uptake, Henn and Hamberger (1971) also reported that glia-enriched cell fractions take up the monoamines norepinephrine, dopamine, and serotonin, albeit much less well than GABA. Uptake of all three monoamines was subsequently demonstrated with astrocytes in primary culture. Norepinephrine and serotonin uptake by cultured astrocytes appear to be very similar to uptake by neurons, as each is highly Na^+-dependent and inhibited by appropriate drugs with appropriately low IC_{50}'s (Ritchie et al., 1981; Tardy et al., 1982; Kimelberg and Pelton, 1983; Kimelberg and Katz, 1985, 1986). Cultured astrocytes also take up dopamine, but, in this case, Na^+ dependency was only observed with astrocytes cultured from hippocampus, and even then Na^+-independent uptake accounted for 70% of total uptake (Kimelberg and Katz, 1986). Astrocytes cultured from corpus striatum and cerebral cortex also took up dopamine, but the uptake was Na^+-independent.

Monoamine uptake by cultured astrocytes is extremely low in comparison to glutamate uptake. The rate of serotonin uptake, for example, is <0.1% of the rate of glutamate uptake, and the other monoamines are accumulated at slightly slower but similar rates (Kimelberg and Katz, 1986). Autoradiographic experiments have demonstrated that most of the astrocytes in the cultures take up [^{3}H]serotonin (Kimelberg and Katz, 1985; Katz and Kimelberg, 1985; Amundson et al., 1992), indicating that uptake is not due to a small population of highly active serotonin-accumulating astrocytes. Thus, the contrast in the rates of serotonin and glutamate uptake appears to be due to differences in the expression of the transporters rather than differences in the number of astrocytes that take up the transmitters.

The rate of astrocytic serotonin uptake is about an order of magnitude slower than that of brain slices (Katz and Kimelberg, 1985). Similarly, the rate of astrocytic dopamine uptake is about two orders of magnitude slower than synaptosomal uptake, and astrocytic norepinephrine uptake is one-third of synaptosomal uptake. (Pelton et al., 1981). Although such comparisons seem to imply that the astrocytic uptake of monoamines is of minor importance, the low transport capacity of cultured astrocytes may not reflect the actual transport capacity of the cells *in situ* but may be a result of growing the cells in highly purified cultures. Furthermore, local rates of uptake by astrocytes *in situ* could be considerably greater if the transport systems are localized in processes, as the GABA and glutamate transporters appear to be (see above).

The metabolism of monoamines is complex and leads to a variety of oxidized, deaminated, methylated, and sulfated products. Two of the principal enzymes of monoamine degradation, monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT), have been shown to be present in glia *in situ* by immunocytochemistry (Kaplan et al., 1979; Levitt et al., 1982; Westlund et al., 1985), and also are present in cultured astrocytes (Hansson, 1984). Cultured astrocytes also contain the enzyme responsible for sulfate conjugation (Yu and Walz, 1985). In keeping with these observations, cultured astrocytes convert norepinephrine to methoxyhydroxyphenylglycol and convert dopamine to dihydroxyphenylacetic acid and homovanillic acid, the expected products (Pelton et al., 1981). The metabolism of the monoamines is sufficiently rapid that the uptake of [^{3}H]norepinephrine and [^{3}H]dopamine is enhanced substantially by inhibitors of MAO and COMT, indicating that the cells rapidly degrade the transmitters and release the metabolites to the medium. This apparent increase in uptake resembles the apparent stimulation of GABA uptake by inhibitors of GABA-transaminase (see above). In both cases, the glia contain virtually no endogenous transmitter, so there is no isotopic dilution of the newly transported transmitter, and the labeled compounds are rapidly degraded.

Adenosine and Adenosine Triphosphate

Cultured astrocytes actively transport and metabolize adenosine (Hertz, 1978; Matz and Hertz, 1989, 1990). The K_m for adenosine uptake by astrocytes (3.4 μM) reportedly is lower than that for brain slices (19 μM), while the V_{max} is about twice that for brain slices. Astrocytes appear to metabolize adenosine by two pathways. In the first pathway, adenosine is converted to adenosine monophosphate (AMP) by adenosine kinase and thus enters the adenine nucleotide pool. In the second pathway, aden-

osine is degraded to inosine by adenosine deaminase. Much of the inosine rapidly appears in the culture medium of astrocytes, a finding that led Matz and Hertz (1989) to suggest that astrocytes possess an ectoenzyme that deaminates adenosine. This point does not appear to have been pursued further.

Adenosine uptake also serves as the final extracellular step for the inactivation of ATP. The initial steps are carried out by ectoenzymes that convert ATP to adenosine diphosphate (ADP) and AMP, and ultimately to adenosine. Although an ectoATPase has been found in cultures of mouse brain glia (Trams and Lauter, 1978) there appear to be no reports that other ectoenzymes of the pathway, such as ATP:AMP phosphotransferase, are present in glial membranes.

Histamine

Relatively little is known about the inactivation of transmitter histamine in brain (Hough, 1988; Schwartz et al., 1991). The major metabolic route of histamine inactivation is thought to be methylation by histamine-*N*-methyltransferase, as brain appears to contain little or no diamine oxidase (Schwartz et al., 1991). A high-affinity but low-capacity transport system for histamine has been found in an astroglia-enriched cell fraction (Rafalowska et al., 1987) and in cultured glia from chick cerebral hemispheres (Huszti et al., 1990). Each of these preparations also contained histamine-*N*-methyltransferase, and the chick glia were able to metabolically degrade histamine to N^{τ}-methylhistamine and N^{τ}-methylimidazoleacetic acid. Histamine uptake by chick glia is inhibited by 2-methylhistamine and 4-methylhistamine, an effect that was attributed to inhibition of histamine-*N*-methyltransferase. This result and interpretation contrast with the results of numerous studies showing that the uptake of GABA and the monoamines is increased when the degradation of these compounds is inhibited. It is notable that the 2- and 4-*N*-methylhistamine compounds are agonists at the H1 and H2 receptors, respectively.

N-Acetylaspartylglutamate

There has been considerable interest in the possibility that *N*-acetylaspartylglutamate (NAAG) is a transmitter in brain, but conclusive evidence supporting this possibility has been elusive. A peptidase that hydrolyzes NAAG to *N*-acetylaspartate and glutamate (NAALADase) has been identified and characterized (Slusher et al., 1992). NAALADase immunoreactivity is enriched in structures that contain NAAG, but has not yet been associated with glia (Slusher et al., 1992). Since NAALADase is an ectoenzyme, it was initially thought that it served to inactivate NAAG, but an alternative possibility has been suggested—namely, that NAAG is a glutamate precursor and that NAALADase generates free glutamate from newly released NAAG in the extracellular space (Meyerhoff et al., 1989). A transporter for *N*-acetylaspartate is present in cultured cerebellar astrocytes from rat brain but not in cerebellar granule cells (O. Saab, personal communication). This transporter appears to be specific for *N*-acetylated acidic amino acids and dipeptides (e.g., NAAG) and is distinct from that for glutamate. Although our understanding of the function of NAAG and *N*-acetylaspartate is still rudimentary, astrocytes are clearly involved.

THE ABILITY OF ASTROCYTES TO TAKE UP TRANSMITTERS IS DETERMINED BY THE KINETICS AND ENERGETICS OF TRANSPORT SYSTEMS

The rate of net transport is governed solely by the kinetic properties of a transport system when it is far from equilibrium, but as a transport reaction approaches equilibrium the rate of net transport decreases and the rate of exchange increases until, finally, equilibrium is reached and net transport stops. Most transport systems for neurotransmitters obtain the energy required to move the substrate from the cotransport of ions such as Na^+, K^+, and Cl^- (see Chapters 15 and 54, this volume; Martin, 1992). The glutamate transport system in retinal Müller cells, for example, is thought to involve the inward cotransport of two Na^+ and the outward transport of one K^+ and one OH^- or HCO_3^- per glutamate moved into the cell (Bouvier et al., 1992).

$$Glu_o^- + 2Na_o^+ + K_i^+ + OH_i^- \rightleftharpoons Glu_i^- + 2Na_i^+ + K_o^+ + OH_o^-$$

Because the ions are cotransported, these systems are often electrogenic, that is, the transport reaction involves the net movement of electrical charge across the membrane. As a result, the total energy available to drive transport can depend on the membrane potential as well as on the stoichiometry of the cotransport reaction (Martin, 1992).

A transport reaction can reach equilibrium because it generates a large transmembrane gradient of neurotransmitter or because changes in the ion gradients or membrane potential reduce the driving force available to drive transmitter uptake. For example, glutamate transport by Müller cells is highly

sensitive to $[K^+]_o$, and when $[K^+]_o$ is increased the driving force becomes too small to support the existing glutamate gradient and the system runs in reverse, thus transporting glutamate from inside to outside the cell (Szatkowski et al., 1990). A similar reversal of transport has been demonstrated for GABA (Chapter 54, this volume). Given this mechanism it seems possible that under conditions of high neuronal activity when $[K^+]_o$ is elevated the ability of astrocytes to take up and inactivate glutamate might be diminished. It is not known at present whether physiological changes in ion gradients and membrane potential actually do affect the ability of astrocytes to take up transmitters *in vivo*. The possibility that changes in ion gradients will affect the uptake of particular transmitters *in vivo* will depend on the stoichiometry of the transport reaction and the extent to which astrocytes maintain or develop a substantial internal concentration of transmitter. Because astrocytes normally contain extremely low levels of GABA and monoamines, their transport systems do not have to move these compounds up a large gradient, and their ability to take up these compounds might not be greatly affected by changes in ion gradients. In contrast, astrocytes do maintain appreciable concentrations of glutamate and aspartate, so the uptake of these compounds may be more significantly affected by membrane potential and ion gradients.

IS THE ABILITY OF ASTROCYTES TO INACTIVATE TRANSMITTERS PHYSIOLOGICALLY SIGNIFICANT?

The anatomical organization of astrocytes and their capacity to take up and metabolize synaptic transmitters has led to the suggestion of at least four specific functions for astrocytes in synaptic transmission (Koelle, 1950; Henn and Hamberger, 1971; Schousboe and Hertz, 1984). First, an astrocyte could help to terminate synaptic transmission at the individual synapses that it envelops. Second, an astrocyte might protect the synapses that it envelops against inappropriate activation by excess transmitter released elsewhere during either physiological or pathological events. Third, astrocytes could take up and metabolize transmitters and then return transmitter precursors to neurons, as in the glutamate-glutamine cycle, and finally, astrocytes might help to regulate synaptic transmission. In the last case, the term "regulate" has not been precisely defined, but seems to contain the idea that astrocytes do not merely intercept and degrade excess transmitter, but actually have an active function in determining the outcome of synaptic events. These ideas are not mutually exclusive, and most of the available evidence can be used to support more than one of them.

Several lines of evidence indicate that the ability of glia to inactivate synaptic transmitters is physiologically significant. First, the ability of astrocytes to take up and metabolize transmitters is not an artifact of tissue culture, but is characteristic of glial cells *in situ*. Uptake by glia *in situ* has been demonstrated by autoradiography and immunocytochemistry for GABA, glutamate, and serotonin (Iversen and Kelly, 1975; Garthwaite and Garthwaite, 1985; Kimelberg, 1986; Storm-Mathisen et al., 1986; Palmi et al., 1991; Anderson et al., 1992). As noted above, glia *in situ* also contain enzymes that degrade acetylcholine, GABA, glutamate, the catecholamines, and serotonin. The observation that metabolic inhibitors enhance the accumulation of radioactively labeled GABA, glutamate, and monoamines indicates that these cells do, in fact, metabolize the transmitters that they accumulate. The relationship of the uptake process to synaptic function is supported by studies of the regional differences in transport properties. Drejer et al. (1982) and Amundson et al., (1992) found that astrocytes cultured from brain regions with a high density of glutamatergic terminals (e.g., neostriatum) had a greater capacity to take up glutamate than did astrocytes from regions with fewer glutamatergic terminals. Similarly, the uptakes of serotonin and the catecholamines differ significantly among astrocytes cultured from different brain regions, although the regional correspondence between serotonin uptake and serotonin fibers was poorer than the correspondence of glutamate uptake with glutamatergic endings (Kimelberg and Katz, 1986; Amundson et al., 1992).

The ability of astrocytes to take up *endogenous* GABA and glutamate is strongly supported by immunocytochemical studies of the distribution of GABA and glutamate during pharmacological and physiological experiments. Inhibition of GABA-transaminase with aminooxyacetic acid or γ-vinylGABA leads to a substantial increase in GABA-like immunoreactivity in glial cells (Storm-Mathisen et al., 1986; Neal et al., 1989; Palmi et al., 1991). Quantitative immunocytochemical studies of the distribution of glutamate and glutamine indicates that the ratio of glutamate to glutamine is greatest in putative excitatory endings and lowest in glial cells (Ottersen et al., 1992). Following ischemia, glutamatelike immunoreactivity rises significantly in hippocampal astrocytes, especially in the mitochondria, while glutamine and taurine remain unchanged or fall somewhat (Torp et al., 1991). In pyramidal cells all three amino acids decrease during ischemia. Because glutamine synthesis requires ATP, the astrocytes would not be able

to convert accumulated glutamate to glutamine during ischemia, and thus astrocytic levels of glutamate would be expected to rise as the transport system accumulates extracellular glutamate. Thus the increased level of glutamate in the astrocytes appears to involve a redistribution of glutamate from the neurons to the astrocytes. Some of the increase in glutamate levels might result from the action of phosphate-activated glutaminase, a mitochondrial enzyme that also is present in glia. Finally, prolonged depolarization of hippocampal slices with high-K^+ medium also reduces the glutamate- and aspartatelike immunoreactivity in hippocampal neurons, while glutamate-, aspartate-, and GABA-like immunoreactivity simultaneously increase in astrocytes (Storm-Mathisen et al., 1986).

A few studies have provided evidence that transmitter-inactivating systems in astrocytes influence neuronal function. In an early study, Desmedt and LaGrutta (1957) compared the effects of selective inhibitors of specific and nonspecific cholinesterase on arousal in the cat's brain and concluded that pharmacological arousal results from inhibition of nonspecific cholinesterase, not the specific cholinesterase. Although they recognized that the nonspecific cholinesterase is a glial enzyme, they did not discuss the implications of their findings for glial function. Bowery et al. (1976) provided evidence that the GABA transport system in the satellite glia of the superior cervical ganglion can influence neuronal activity. During studies of the depolarization of ganglion neurons by β-alanine and GABA, they discovered that the effects of β-alanine were enhanced after the satellite glia were preloaded with GABA. They attributed this effect to elevated extracellular GABA resulting from the inhibitory effect of β-alanine on the reuptake of GABA by the glial transport system. That is, because β-alanine inhibited the uptake of effluent GABA into the glia, extracellular GABA levels rose and the neurons were depolarized. Although the ganglion glia contain little or no GABA *in vivo* and the physiologic function of this mechanism is unknown, these data do indicate that the GABA transport system in the ganglion glia is close enough to the neuron and is active enough to influence neuronal activity. The physiological importance of the glial GABA transport system also is supported by the observation that THPO (4,5,6,7-tetrahydroisoxazolo [4,5c]pyridin-3-ol) and THAO (5,6,7,8-tetrahydro-4H-isoxazolo [4,5c]azepin-3-ol), two relatively specific (but weak) inhibitors of the glial GABA transport system, have anticonvulsant activity (Schousboe et al., 1991).

Thus the current evidence indicates that inactivation of transmitters by astrocytes is physiologically significant in the sense that it does occur, but its exact relationship to synaptic transmission and its quantitative importance is not clear at all. Although the quantitative aspects of the problem are poorly understood, they are not unimportant, as the astrocyte's role would likely be considered minor if it only inactivated a few percent of the transmitter released at a synapse but highly significant if it inactivated, say, half. The exact function and quantitative importance of astrocytic inactivation undoubtedly differs among transmitters and neuronal circuits. It is widely accepted that the glutamate-glutamine cycle is quantitatively important in glutamatergic synaptic transmission (Schousboe and Hertz, 1984), although the exact proportion of glutamate handled by the pathway is unknown. Astrocytically produced glutamine is also a quantitatively important precursor of transmitter GABA (Martin and Rimvall, 1993), but this fact does not imply that astrocytes take up and degrade a large fraction of transmitter GABA in a GABA-glutamine cycle, as astrocytes cannot directly convert GABA to glutamine, and as noted above, GABA uptake by astrocytes is variable and somewhat slower than uptake by cortical neurons. Similarly, astrocytes take up monoamines relatively slowly, an observation that seems to suggest that astrocytes do not play an important role in the inactivation of monoamines. However, such observations may be misleading, as the transport systems may be strategically placed on the surfaces of astrocytes in order to effectively handle the overflow of a particular synaptic transmitter. Studies of transmitter inactivation by glia appear to be entering a new phase, as the genes for some of the transporters have been cloned and new, specific antisera to some of the transporters have been prepared. The advent of these new tools will greatly enhance our ability to investigate this fundamental aspect of glial function.

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49 Radial glial cells: scaffolding for brain construction

PASKO RAKIC

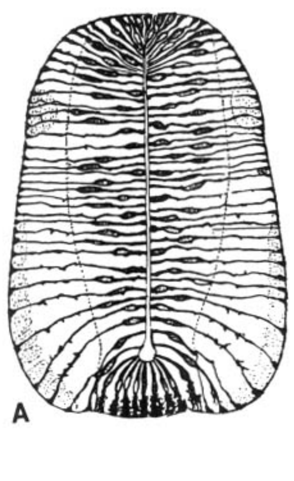
Among the nonneuronal cells that can be encountered in the mammalian brain, none are larger or more fascinating than radial glial cells. Although in most brain regions they are present only transiently, during a short developmental period they play a crucial role in the construction of the nervous system, first, by providing scaffolding for migrating neurons and, second, by participating in the formation of diverse glial cell lineages. The radial glial cell is a maverick among brain cellular elements in that it initially assumes one phenotype and subserves distinct developmental functions and then, after reentering mitotic cycles, it transforms into a morphologically and functionally different phenotype. The diversity of functions and morphogenetic transformations that have been observed in various vertebrate species suggests that this glial cell class may have played a special facilitatory role in the evolution of the central nervous system. This chapter is concerned with radial glial cells and their role in the developing mammalian brain, with particular emphasis on the primate telencephalon that was the focus of research in my laboratory.

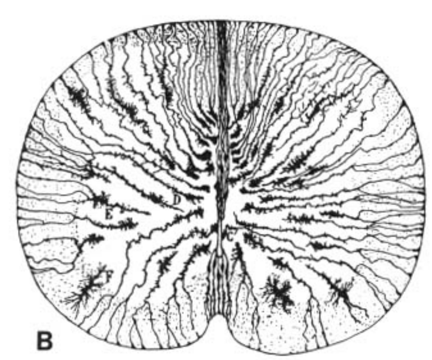
A LITTLE HISTORY

As the name implies, radial glial cells are a specialized nonneuronal cell class belonging to the astroglial cell lineage. They are a morphologically, biochemically, and functionally distinct cell class characterized by an elongated fiber of an even caliber that, initially during development, radially spans the entire cerebral wall from the ventricular to the pial surface. In most mammals, with some notable exceptions, the majority of radial glial cells disappear during late stages of ontogenetic development.

Radial glial cells impregnate remarkably well with most variants of the Golgi silver method and were described in the fetal human brain before the turn of the century (Ramón y Cajal, 1890; Lenhossék, 1895; Retzius 1993; Golgi, 1985). However, distinguishing between immature neuronal and glial cells in the developing nervous system is rather difficult. Ramón y Cajal had a hard time making up his mind as to the nature of "fetal radial cells," but eventually corrected his own initial misperceptions (Ramón y Cajal, 1890, 1909). In his study of the development of the chick embryo, he first thought that the "neuroepithelial" cells that span the entire width of the neural tube (Figure 49-1A) were exclusively neuroblasts, which subsequently would form the ventral and dorsal horns of the spinal cord. Later, however, he recognized the presence of additional "transitional" cells (also described by Lenhossék, 1895), which he designated as precursors of protoplasmic and fibrous astrocytes in the spinal cord (Figure 49-1B), mesencephalon (Figure 49-1C), and telencephalon (Figure 49-1D). Soon after the introduction of the Golgi method, numerous other investigators described elongated "fetal glia" under a variety of terms such as "epithelial cells" (Golgi, 1885; Ramón y Cajal, 1909), "radial cells" (Magini, 1888), "fetal ependymal cells" (Retzius, 1893, 1894), and "spongioblasts" (Lenhossék, 1895; Kölliker, 1896). More recent terms, such as "tanycytes" and "faserglia," have added to the confusion in the literature (see discussion in Schmechel and Rakic, 1979a). To avoid connotations beyond a morphological description, I adopted the term *radial glia* (Rakic, 1971a, 1972). This designation is now generally accepted and will be used throughout this chapter.

The difficulties in identifying immature cells with classical methods, their obscure function, and their transient presence, may be why radial glial cells were neglected soon after discovery. This cell type is rarely mentioned in textbooks of neuroanatomy or neurophysiology published in the first half of this century. However, the introduction of electron microscopy, which revealed the possible role of radial glia in neuronal migration (Rakic, 1971a, 1971b) reawakened the interest of researchers in these cells. More recently, application of immunocytochemistry and the development of *in vitro* systems have permitted the molecular characterization of radial glial cells and provided additional insights into their role





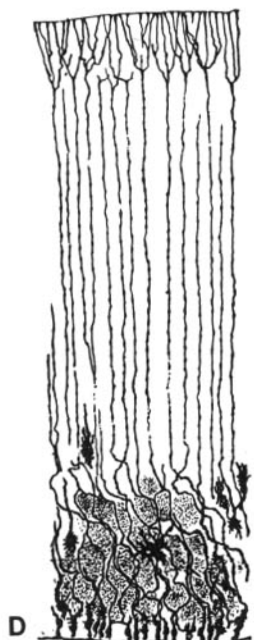


FIG. 49-1. (**A**) Primordial epithelium, including spongioblast (radial glial) cells of the spinal cord of the chick embryonic day 3 of incubation when, according to Ramón y Cajal (1909, Vol. 1, p. 662), they become stainable by the Golgi method. (**B**) Epithelial (radial glial) and displaced astroglial precursors and more mature astrocytes, stained by the Golgi method, in the spinal cord of the newborn mouse. This drawing shows explicitly different phases of morphogenetic transformation of glial cell line. (Ramón y Cajal, 1909, Vol. 1, p. 240). (**C**) Epithelial (radial glial) and (**D**) neuroglial cells of the cerebral cortex in neonatal rabbit stained with the Golgi method. [Adapted from Ramón y Cajal, 1909, Vol. 2, p. 859.]

during development (for review see Cameron and Rakic, 1991; Misson, 1991).

ORIGIN AND PHENOTYPE

Radial glial cells of the mammalian telencephalon, as in most other brain structures, have a distinct bipolar shape. Their spindle-shaped soma-containing nucleus is initially situated near the lumen of the cerebral vesicle with an inner endfoot anchored at the ventricular surface. From the other pole of the soma emanates an elongated fiber that ultimately terminates with a conical endfoot at the pial surface. During early embryonic development, these glial cells are an integral part of the columnar germinal epithelium with outwardly directed processes that stretch across the full thickness of the neural wall (Sidman and Rakic, 1973). As the cerebral wall becomes wider and more complex in configuration, radial fibers further elongate and curve, retaining an attachment to both the ventricular and pial surfaces. In the large human fetal brain, the length of a radial glial fiber may attain 15 to 20 mm, which makes them the largest and longest glial cells recorded (Rakic, 1984). Although the elongated shafts of radial glial fibers initially have smooth surfaces, during the midgestational period they develop numerous lamellate expansions that protrude laterally from the segment passing through the intermediate zone (Rakic, 1972; Schmechel and Rakic, 1979a).

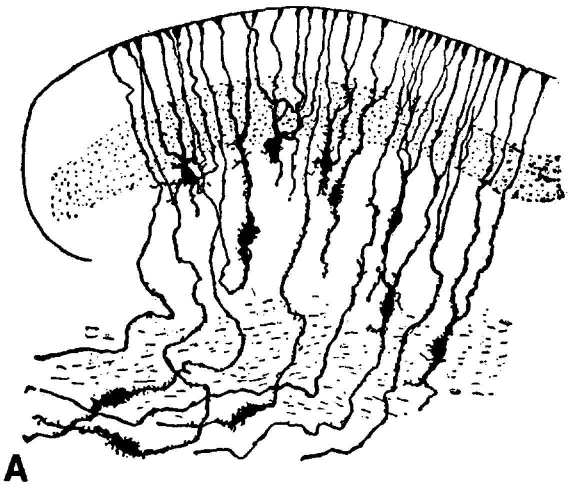
Golgi, electron microscopic, and immumocytochemical studies helped to determine the main cytological characteristics of radial glial cells in the mammalian telencephalon, which in essence are as follows (see Chapter 4, this volume):

1. An endfoot situated at the ventricular surface and connected with the adjacent cells by tight junctions
2. An ovoid nucleus with homogeneously dispersed chromatin

3. A spindle-shaped soma situated within the proliferative, ventricular, or subventricular zones
4. An elongated, radially oriented fiber that contains attenuated mitochondria, sheaves of 24-nm microtubules, an electronlucent cytoplasmic matrix, and, at later stages, an abundance of 9-nm intermediate filaments
5. An immunoreactivity to astroglial cell-specific markers, such as glial fibrillary acidic protein (GFAP) and to several other radial glia-specific antibodies (see below)
6. A terminal endfoot at the pial surface rich in glycogen granules (these endfeet form a continuous sheet at the outer cerebral surface, the so-called limiting membrane, which is coated externally by a basal lamina rich in collagen and laminin)

There are several morphological variations of the basic radial glial cell phenotype. For example, as suggested by Ramón y Cajal (1909), Bergmann glial cells of the mammalian cerebellum can be considered modified radial glial cells. The transitional forms of Bergmann glial cells as they evolve from the primitive radial glial cells during embryonic development are illustrated in Figure 49-2A. In primates, transformation from the radial glial phenotype into Bergmann phenotype occurs between 90 and 120 embryonic days (Rakic, 1975) and proceeds a major wave of granule cell migration from the external granular layer across the molecular layer. A similar morphogenetic transformation seems to occur during the course of vertebrate evolution. Thus, in adult amphibian and reptilian cerebellum, Bergmann glial cells retain contact with both ventricular and pial surfaces and have the general spindle-shaped morphology characteristic of the transient telencephalic radial glial cell (Figure 49-2B).

Both Golgi and immunocytochemical analyses of the primate telencephalon, using various glial cell-specific antibodies, show that radial glial cells are probably the earliest class of nonneuronal cells to appear in all major regions of the developing mammalian brain (Rakic, 1972; Levitt et al., 1981, 1983). For example, glial fibrillary acidic protein (GFAP)-positive radial glial cells were detected in the fetal brain of humans and nonhuman primates during the first quarter of gestation (Levitt al., 1983; Choi, 1986). However, it is possible that this nonneuronal cell line is established during even earlier developmental stages, since commitment to the glial cell lineage does not necessarily begin with the expression of GFAP (Cameron and Rakic, 1991). Initial discovery of a separate population of GFAP-positive dividing cells in the germinal ventricular zone (Levitt et al., 1993) indicated that astrocytic



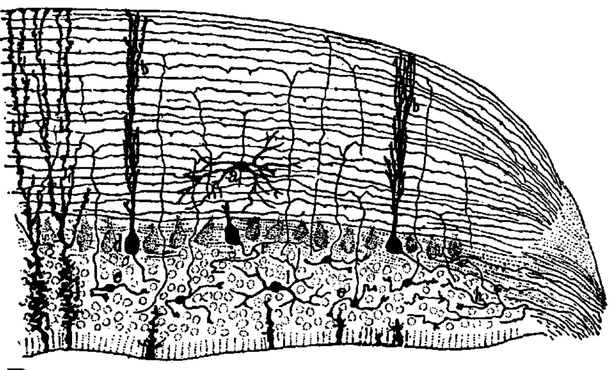


FIG. 49-2. (A) Sagittal section of the cerebellum of the newborn mouse stained with the Golgi method illustrates the transformation of epithelial (radial glial) cells into Bergmann glial cells (Ramón y Cajal, 1911, Vol. 2, p. 105). (B) Frontal (coronal) section of the adult reptilian cerebellum stained with the Golgi method shows that in many submammalian species the cerebellum does not contain Bergmann glial cells but rather radial glial cells, which remain present throughout life. [Adapted from Ramón y Cajal, 1909, Vol. 2, p. 77.]

glial cell lines diverge within the population of dividing precursor cells (Figure 49-3A). Most of the cells committed to the glial line retain their capacity to divide in the fetus (Schmechel and Rakic, 1979b) and infancy (Misson, 1991).

The developmental history of a radial glial cell line in the mammalian cerebrum has been clarified using a combination of methods, including sets of new mono- and polyclonal antibodies (for review see Cameron and Rakic, 1991). The presently available data indicate that progenitorlike or primary radial glial cells, which in nonprimates do not express the GFAP, are the earliest of the telencephalic glial cell sublineages to be identified by morphological and immunological criteria. This system of radial glial

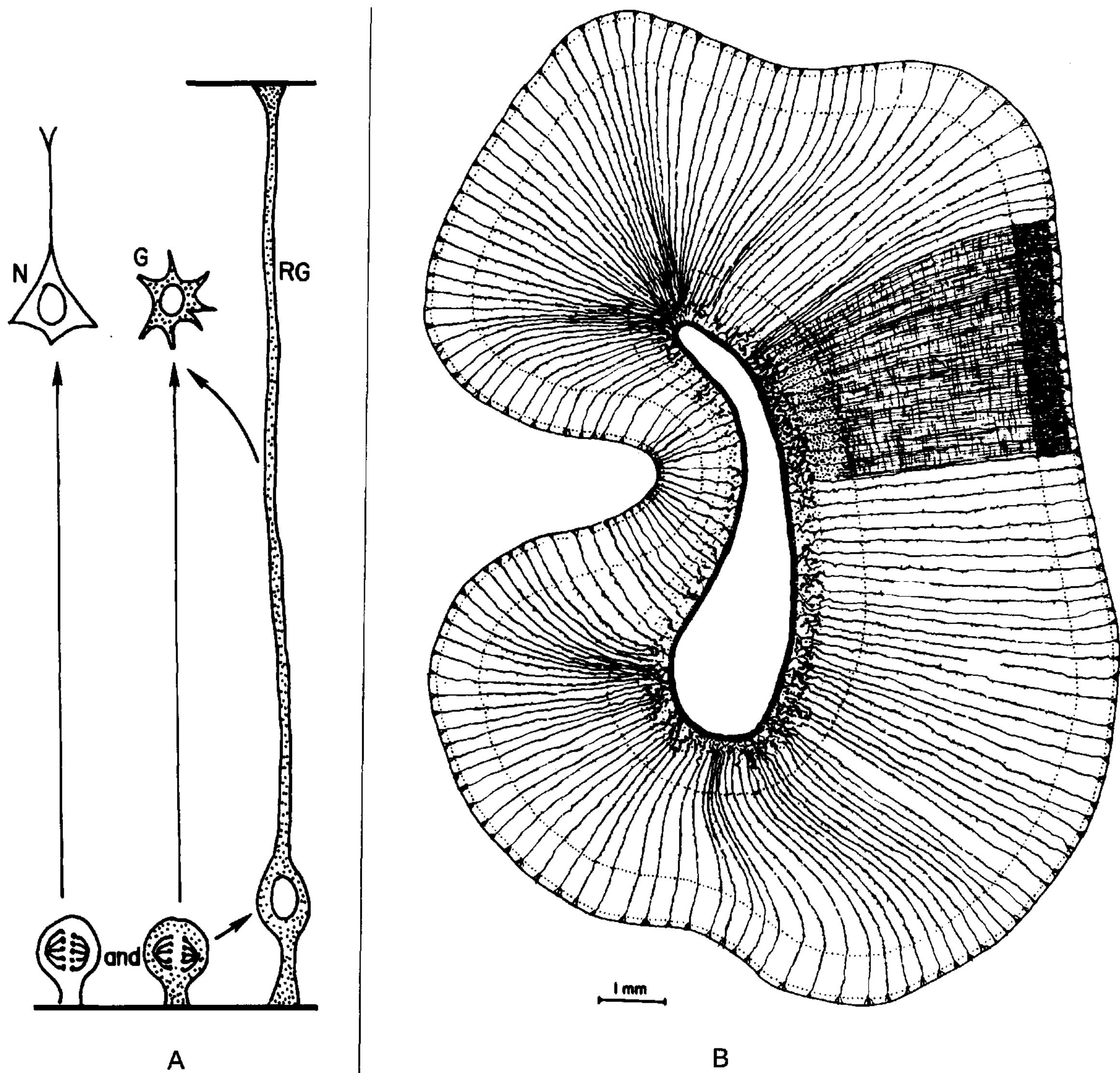


FIG. 49-3. (A) Dual cell line origin and concept of morphogenetic transformation of radial glial cells into astrocytes in the fetal monkey cerebrum based on Golgi, electron microscopic, and immunocytochemical analyses. GFAP-negative and GFAP-positive (*stippled*) dividing cells coexist throughout the period of neurogenesis. The GFAP-positive cells initially produce radial glial cells (*RG*); later, either directly or indirectly (*arrows*), they generate astrocytes (*G*) and various specialized astrocyticlike cells. Neurons (*N*) or perhaps also some nonneuronal cells originate from GFAP-negative precursors. [From Rakic (1981), with permission.] (B) Camera lucida drawing of a coronal section of a Golgi-impregnated occipital cerebral lobe in a 97-day-old monkey fetus. The incipient calcarine fissure is at the left. The radial glial cell somas are situated close to the cerebral ventricle and their elongated fibers extend to the 3 to 5 mm distant pial surfaces where they divide into several branches, each terminating with an endfoot. [Adapted from Rakic (1972), with permission.]

cells appears to be assembled in the telencephalon prior to the earliest migratory wave of postmitotic neurons in all species examined (Levitt et al., 1981, 1983; Rickmann et al., 1987; Gadisseux et al., 1989; Gressens et al., 1993). The original concept of two separate precursor cell lines and two alternative morphogenetic pathways for production of astrocytes in the primate is illustrated schematically in Figure 49-3A. Use of the retrovirus gene transfer method to label progeny of cells confirms the existence of separate glial cell lines in the developing mammalian telencephalon (Luskin et al., 1988). The emergence of the radial glial cell lineage and its differentiation from primitive neuroepithelial cells committed to neuronal cell precursors is also indicated by the expression of the radial glial cell-specific antigens RC1 and RC2 and the absence of immunoreaction with neurofilament (Frederiksen and McKay, 1988; Misson et al., 1988a, 1988b; Culican et al., 1990; Edwards et al., 1990). Although these two cell populations continue to coexist during the entire period of corticogenesis, their proportion changes in

harmony with the appearance of increasing increments of neuronal and astroglial cells (Levitt et al., 1983). At present, it is unclear whether the glial-specific RC1 and RC2 antigens are expressed in the multipotential glial progenitor cell as well as primary radial glial cells (Cameron and Rakic, 1991). This uncertainty is reflected in the cell lineage diagram (Figure 49-4).

Glial fibrillary acidic protein is present in the cytoplasm of glial cells in all vertebrate species, exclusive of cyclostomes, and provides a definitive marker of the astrocytic cell phenotype, including radial glial cells (Onteniente et al., 1983; Dahl et al., 1985; Bignami, 1991). The intermediate filament vimentin is also a very useful marker for the identification of radial glial cells, since the adjacent neuroepithelial cells are vimentin-negative. The phenotype of the primary radial glial cell in rodents is characterized by immunoreactivity to RC1, RC2, vimentin, Rat 401, and Ran-2. However, antigenic properties are changed during the emergence of a secondary phenotype as revealed by a substitution in the intermediate filament protein composition: vimentin to GFAP (Bovoienta et al., 1984; Pixley and De Vellis, 1984; Rickmann et al., 1987; Hutchins and Casagrande, 1989; Voigt, 1989). Similar studies of biochemical composition have not been carried out in primates.

FUNCTION AND PATHOLOGY

The striking geometrical regularity of radial glial cells in the developing central nervous system suggested that these cells may serve as a scaffolding for fragile embryonic neural tissue (Ramón y Cajal, 1909). The relationship between radial glial cells and migrating neurons was suggestive in classical light microscopic preparation (Figure 49-5) (Rakic, 1972). However, only resolution obtainable by a transmission electron microscope could reveal the close apposition between postmitotic migrating neurons and radial glial cells (Rakic, 1971a, 1972). This relationship is particularly explicit in developing cerebellar cortex between Bergmann glial fibers and granule cells (Figure 49-6) (Rakic, 1971). Several lines of evidence from both *in vivo* and *in vitro* analyses confirmed that contact interaction between the surface of radial fibers and migrating neurons plays a crucial role in the selection of the migratory pathway as well as in generating signals for displacement and stopping the movement of neurons (Caviness and Rakic, 1978; Gadisseux and Evrard, 1985; Rakic, 1988b; Edvards et al., 1990; Hatten, 1990; Misson, 1991; Komuro and Rakic, 1993). In terms of orientation and directionality of cell movement, migration can be classified into radial (proceeding from the ventricular to the pial surface) and tangential (running parallel to the brain surface). With regard to pathway selection, migrating neurons fall into three major categories: (1) gliophilic cells, which follow elongated glial fibers and bypass neurons that may be lying within their trajectory; (2) neurophilic cells, which follow neuronal, particularly axonal, surfaces and bypass glial shafts; and (3) biphilic cells, which display temporal or regional affinities toward either glial or neuronal surfaces (Rakic, 1985, 1990).

The gliophilic migration is particularly prominent during formation of the neocortex (Rakic, 1972) but occurs also in the developing hippocampus (Nowakowski and Rakic, 1978; Eckenhoff and Rakic, 1984). In each case electron microscopic analysis of closely spaced serial sections showed that with some notable exceptions (Rakic et al., 1974; Nowakowski and Rakic, 1978), typical migrating neurons in the primate cerebral wall adhere intimately and continuously to the neighboring glial fibers (Figure 49-7). In spite of clearly defined columns of migrating neurons aligned with radial glial cells, some postmitotic cells in the cerebral wall do not respect radial constraints (e.g., Tan and Breen, 1993). Although their identity is not clear, it appears that at least some of the tangentially migrating cells belong to a separate subclass, as observed in mouse telencephalon (Luskin et al., 1992) or in avian optic tectum (Gray and Sanes, 1991; Martinez et al., 1992). Many of the tangentially moving cells within the cerebral wall do not ever enter the neocortex (DeDiego et al., 1994). These findings suggest that some postmitotic cells may be neurophilic and are actually prespecified to move perpendicularly to the glial scaffolding along axonal pathways to subcortical structures. In spite of these exceptions, a majority of postmitotic cells in the mammalian cerebral wall become gliophilic and remain closely associated with adjacent glial cells while migrating to the cortical plate. This relationship is particularly prominent in primates, where migratory pathways are much longer and both radial and laminar organization is more pronounced than in subprimate species (Sidman and Rakic, 1973). See also Figures 49-3B and 49-7.

The affinity between gliophilic neuronal and radial glial fiber surfaces was implied by the failure of migration cells to follow any of a myriad of differently oriented cellular processes that they encounter during the journey to the cortex (Rakic, 1972). The leading process of migrating neurons morphologically polarized and contoured to dimensions of the radial glial fibers was suggestive of differential cell

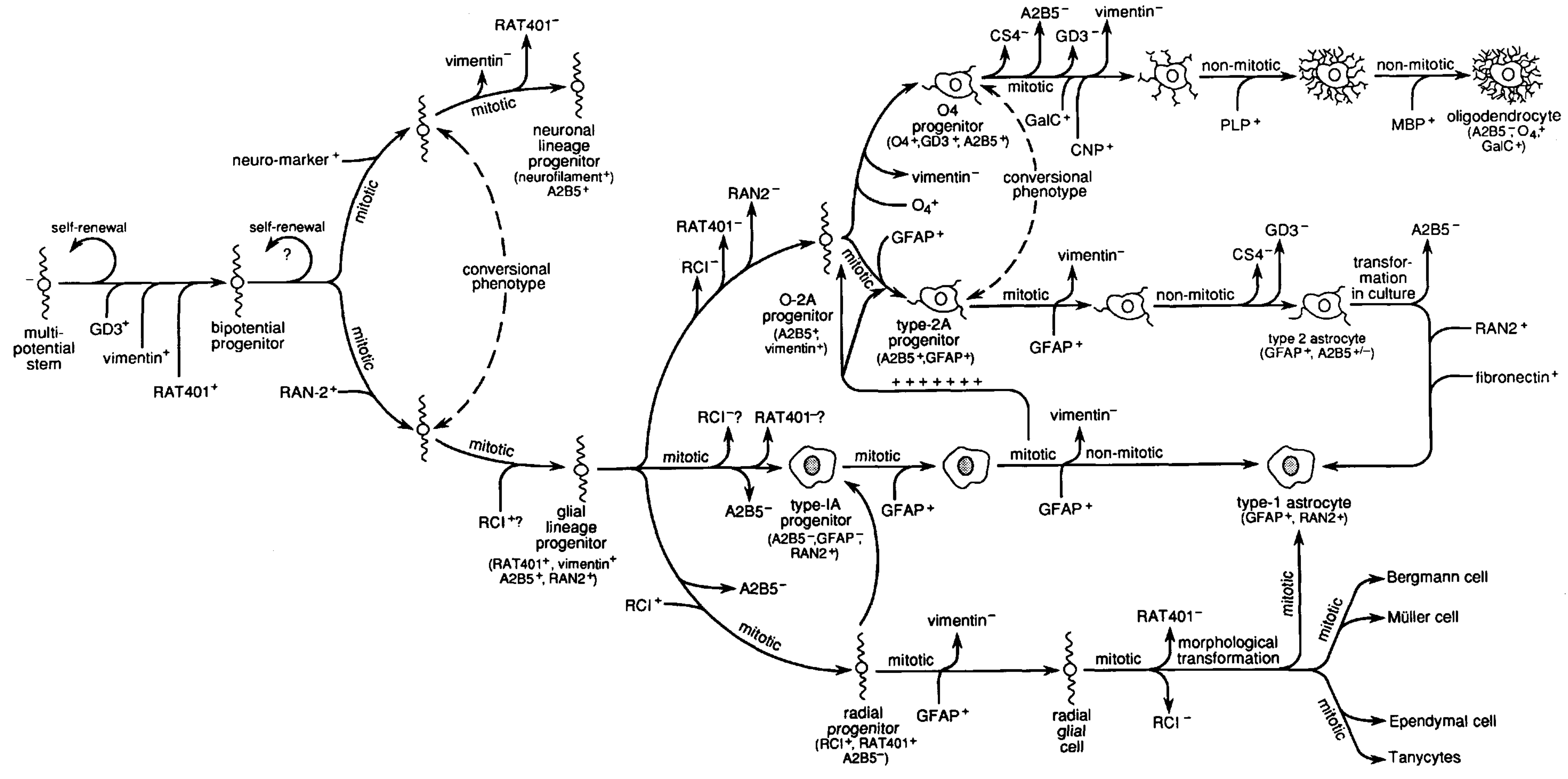


FIG. 49-4. Possible glial cell lineages that occur during the development of the cerebral cortex. In the past two decades we have witnessed introduction of a large number of molecular markers specific for various subtypes of glial cells. The acquisition or loss of antigenic components that accompany the progressive maturation of individual cell types are indicated by arrows with (+) or (−) signs. [Adapted from Cameron and Rakic (1991), with permission.]

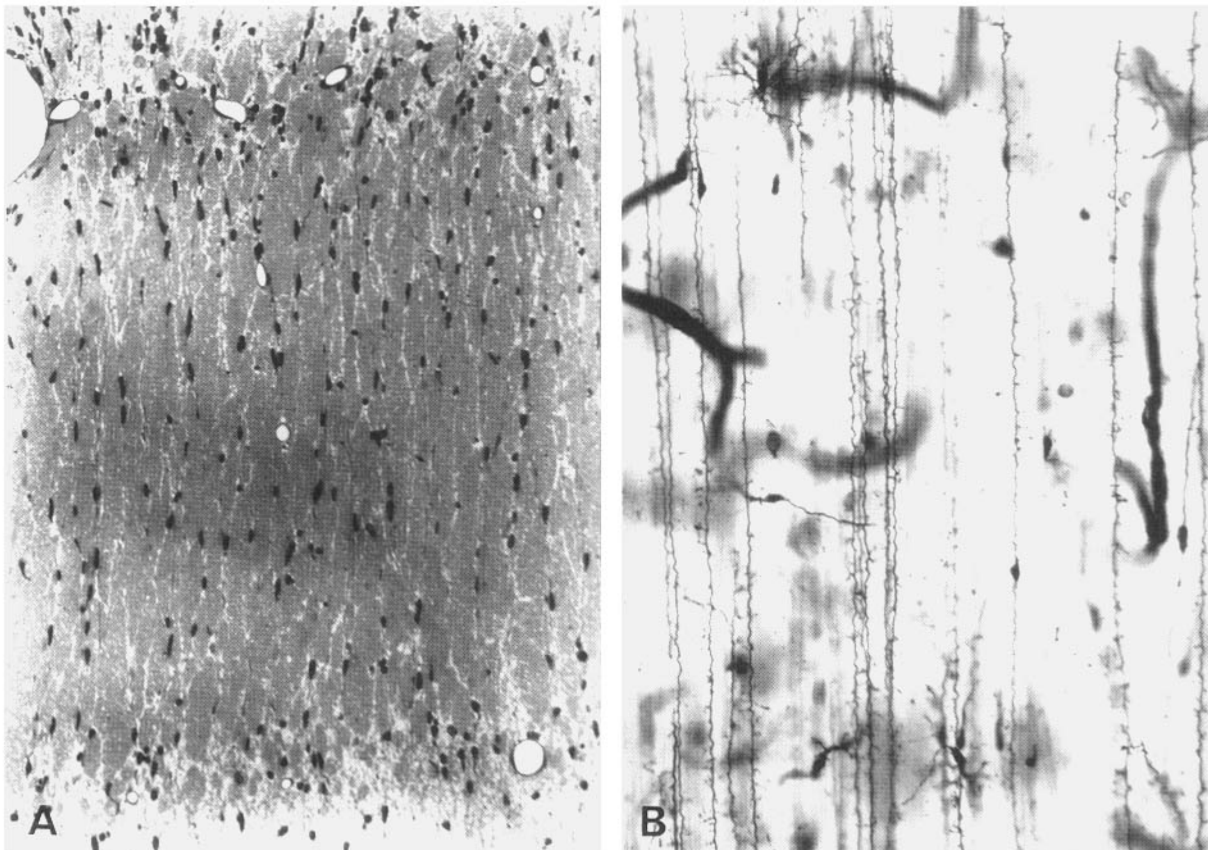
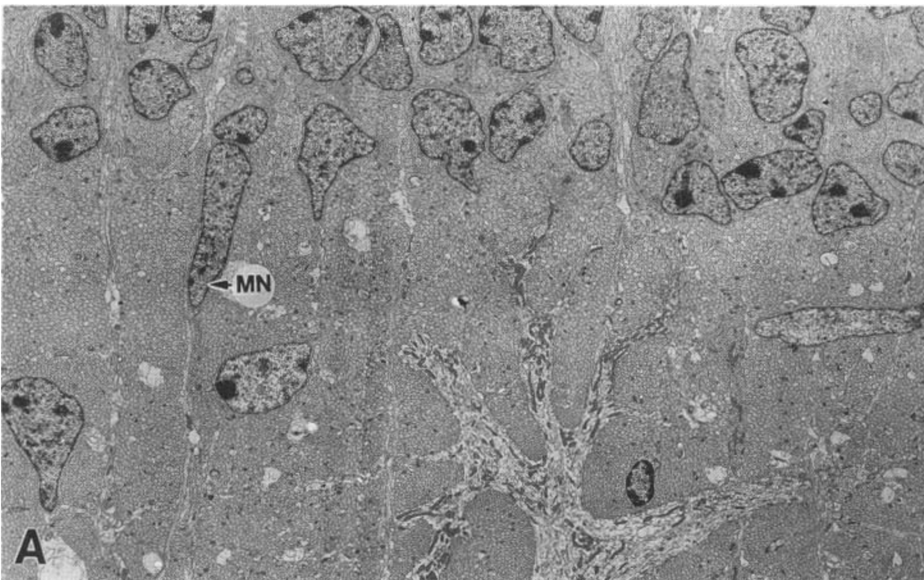


FIG. 49-5. (A) Photomicrograph of the toluidine blue stained section across the intermediate zone in the monkey cerebral wall at midgestation. Note cohorts of migrating neurons deployed in the separate streams running from the proliferative ventricular zone situated 1000 μm below to the cortical plate 2000 μm above. (B) Golgi impregnated section at the corresponding level of the fetal cerebral wall exposes elongated shafts of radial glial fibers and an occasional bipolar migratory neuron. [Adapted from Rakic (1990), with permission.]

adhesion (Figure 49-7). Thus, fetal radial glial fibers provide transient scaffolding and impose radial constraints by providing guidelines that facilitate neuronal migration in the expanding cerebral wall.

Cerebellar granule cells can be classified as the biphilic category of migrating cells because their single descending process containing soma follows exclusively Bergmann glial fibers while their two horizontal neurites grow along parallel axonal fibers belonging to previously generated granule cells (Rakic, 1985). Shortly after its final cell division in the external granular layer, a newly generated granule cell takes a position in the deep part of the external granular, contacting a Bergmann glial fiber (cell 1 in Figure 49-8). It then transiently assumes a bipolar shape (cell 2) by emitting two horizontal cytoplasmic processes that run in the longitudinal plane of the cerebellar folium, at right angles to the growing Purkinje dendritic trees (Ramón y Cajal, 1909). These horizontal processes extend exclusively along the surface of parallel (axonal) fibers (Rakic, 1971b). Next, the granule cell becomes tripolar by forming a third, vertical cytoplasmic process, which elongates in close apposition to the shaft of Bergmann glial cells (cell 3). When the descending process reaches the appropriate length, the nuclear part of the granule cell becomes translocated within its volume. As a result, the entire soma passes across the complex and synaptically interconnected molecular layer. Several granule cells in succession follow the same Bergmann glial guide, as described for the developing cerebral cortex (Rakic, 1971b).

A change in position and shape of the cerebellar granule cells provides an example of the significance that cooperation between neuronal and glial cells has for the development of complex cellular and synaptic organization of a given brain structure. The migration is an essential prerequisite for forming granule cell shape: the horizontal and vertical segments of the cell trailing behind the nuclear region of the granule cell become the parallel fiber and vertical shaft, respectively. When the granule cell body



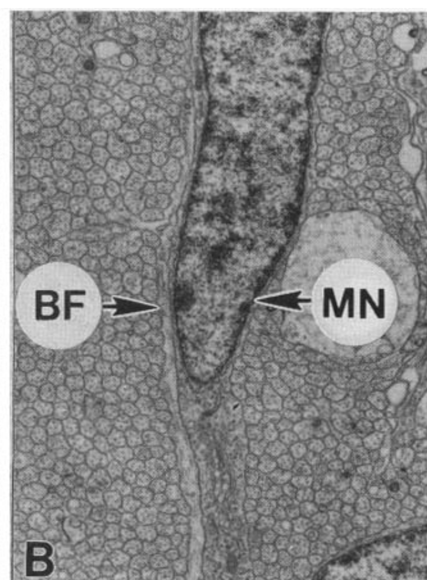


FIG. 49-6. (**A**) Low-power electron micrograph of the 135-day-old monkey fetus showing bipolar migrating neuron (*MN*) descending from the external granular layer situated at the top of this field, to the internal granule layer situated below. (**B**) An enlargement of the migrating neurons (*MN*) to show that its leading process is closely aligned with an electronlucent shaft of the Bergmann glial fiber (*BF*) as the cell penetrates the densely packed and already synaptically interconnected neuropil of the molecular layer. The migrating neurons move selectively along the surface of the glial fiber, although it simultaneously contacts a myriad of axons and other cell processes.

reaches Bergmann cell soma at the level of the Purkinje cell layer, it leaves its glial guide and takes up its permanent position in the internal granule layer (Figure 49-8 cells 5, 6, and 7). It then emits 3 to 5 short dendrites that contact the mossy fibers that originate in the brainstem. The position in the molecular layer where granule cells differentiate from round into bipolar form determines the depth of the prospective parallel fibers, so that early generated parallel fibers remain near the Purkinje cells, while those situated closest to the pial surface form the last, as evidenced by examining the position of parallel fibers belonging to cells 2, 3, 6, and 7 in Figure 49-6.

Selective displacement of migrating neurons along the glial surface can be explained by the differential adhesion mediated by several classes of recognition and/or adhesion molecules (Edelman, 1980; Rakic, 1981, 1985; Hatten, 1990; Rakic et al., 1994). So far, most of the identified molecules are present on the neuronal, or on both neuronal and glial, cell surface (e.g., Edmonson et al., 1984; Edelman and Crossin, 1991; Fishel and Hatten, 1991). Only recently two new membrane proteins, that may be involved in neuronal migration, were found transiently on the surface of the radial glial cells (Cameron and Rakic, 1994). These polypeptides are strategically localized in a form of well-defined junctions between the soma of migrating neurons and radial glial fibers. One important feature of potential recognition molecules is that their expression should be transiently present on the glial surface. These polypeptides are indeed expressed in the telencephalon and cerebellum only during the period of neuronal cell migration (Cameron and Rakic, 1994). In the cerebellum, where Bergmann glial cells persist in the adult, several other of its cytoplasmic and surface properties also change from the embryonic to adult form. However, a recent study shows that adult Bergmann glial can be induced to reexpress at least some of these immature components, including nestin, an intermediate filament normally present only during the active phase of neuronal migration (Sotelo et al., 1994). Such reexpression allows Bergmann fibers in the adult cerebellum to serve as conduits for implanted neuroblasts.

Recognition of, and attachment to, the appropriate substrate surface is not a sufficient condition to translocate a cell nucleus and the adjacent cytoplasm across densely packed neural tissue. Recent studies indicate that glial cells, though lacking the high excitability of neurons, are intimately associated with neurons through an exchange of signals regulated by the same species of ion channels (Barres, 1991). During development, the motility of migrating neurons seems to depend critically on Ca^{2+}-mediated assembling of cytoskeletal proteins regulated by a combination of several specific ion channels (Komuro and

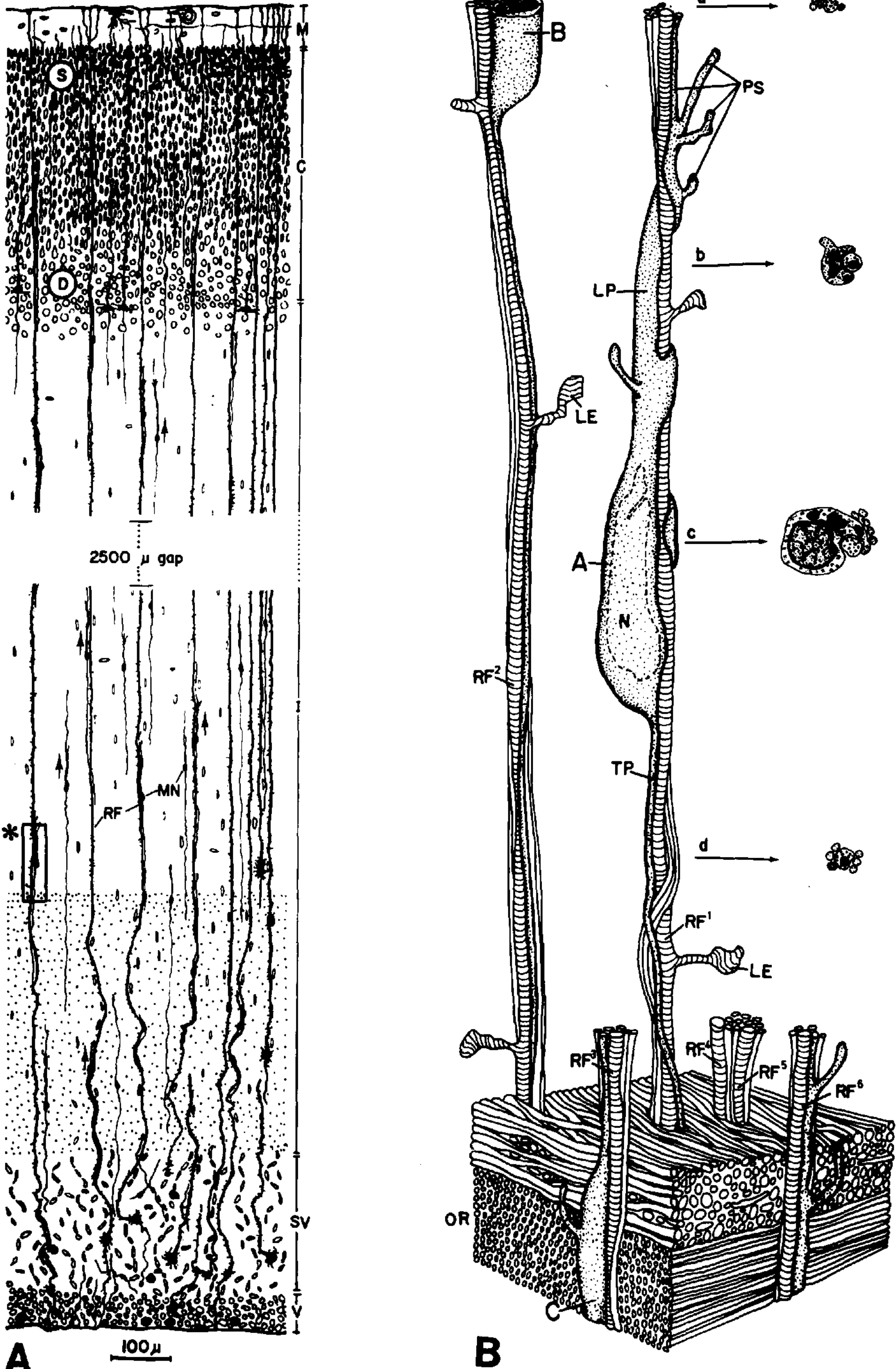


FIG. 49-7. (A) Camera lucida drawing of the occipital lobe of cerebral wall of the monkey fetus at midgestation. Composite illustration is derived from Golgi section (*black profiles of cell images*) and from adjacent section counterstained with toluidine blue (*outline profile of cell nuclei*). The middle 2500 μm of the intermediate zone, similar in structure to the sectors drawn, is omitted. The rectangle marked with an *asterisk* shows the approximate position of the three-dimensional reconstruction in Figure 49-7B. *C*, cortical plate; *I*, intermediate zone; *M*, molecular layer; *MN*, migrating neuron; *RF*, radial fiber; *SV*, subventricular zone; *V*, ventricular zone. (B) Three-dimensional reconstruction of migrating neurons, based on electron micrographs of semiserial sections of the occipital lobe of the monkey fetus. The reconstruction was made at the level of the intermediate zone indicated by the rectangle in Figure A. The subventricular zone lies some distance below the reconstructed area, whereas the cortex is more than 1000 μm above it. The lower portion of the diagram contains uniform, parallel fibers of the optic radiation (*OR*) and the remainder is occupied by more irregularly disposed fiber systems; the border between the two systems is easily recognized. Except at the lower portion of the figure, most of these fibers are deleted from the diagram to expose the radial fibers (*striped vertical shafts* RF_{1-6}) and their relations to the migrating cells A, B, and C, and to other vertical processes. The soma of migrating cell A, with its nucleus (*N*) and voluminous leading process (*LP*), is situated within the reconstructed space, except for the terminal part of the attenuated trailing process and the tip of the vertical ascending pseudopodium. Cross sections of *cell A* in relation to the several vertical fibers in the fascicle are drawn at levels *a–d* at the right side of the figure. The perikaryon of *cell B* is cut off at the top of the reconstructed space, whereas the leading process of *cell C* is shown just penetrating between fibers of the optic radiation (*OR*) on its way across the intermediate zone. *LE*, lamellate expansions; PS, pseudopodia. [Adapted from Rakic (1972), with permission.]

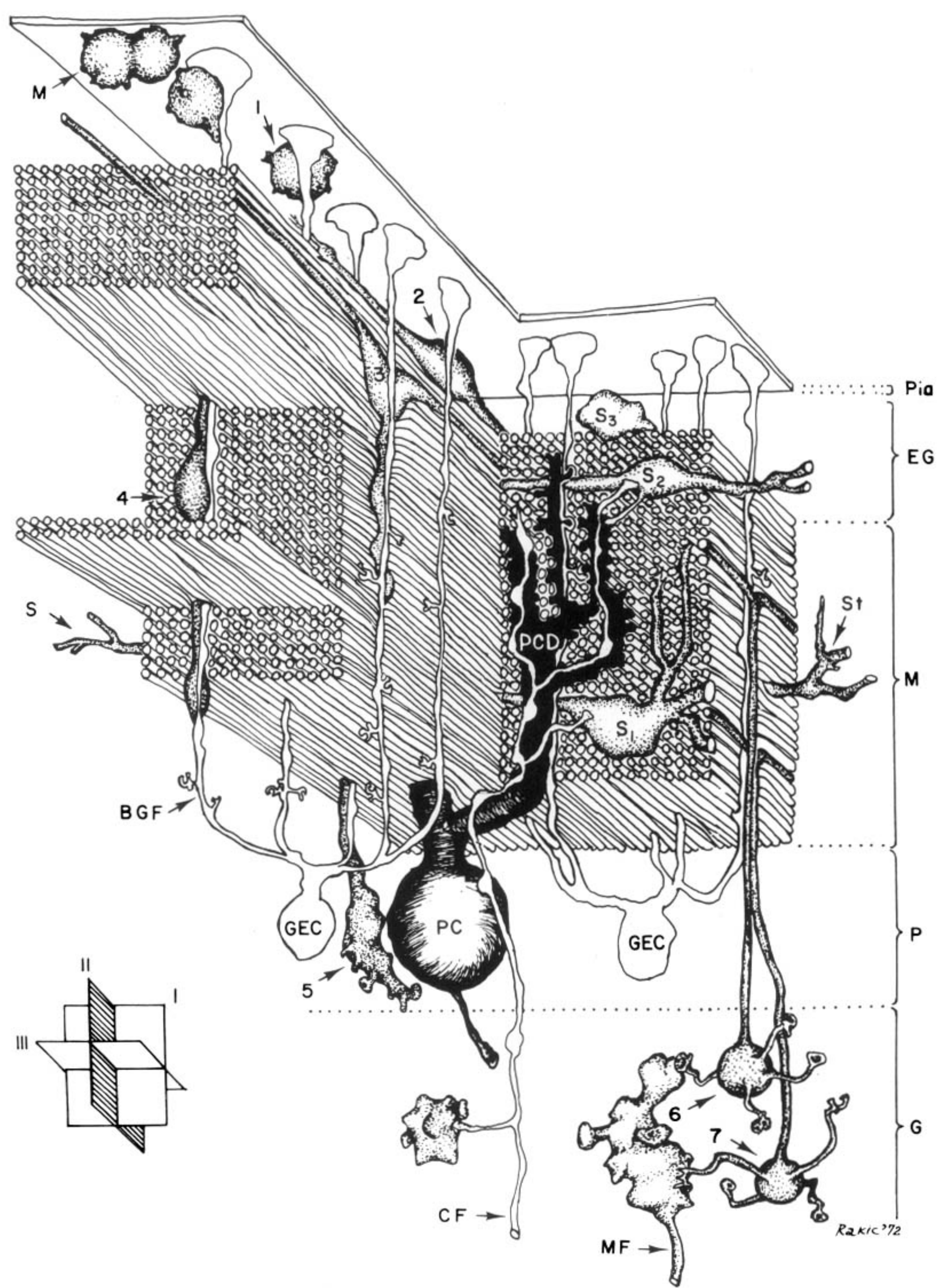


FIG. 49-8. Four-dimensional (time and space) reconstruction of the developing cerebellar cortex in the rhesus monkey. The geometric figure in the lower left corner indicates the orientation of the planes: *I*, transverse to the folium (sagittal); *II*, longitudinal to the folium; *III*, parallel to the pila surface. On the main figure, the thicknesses of the layers are drawn in their approximately true proportions for the 138-day-old monkey fetus, but the diameters of the cellular elements, particularly the parallel fibers, are exaggerated to make the reconstruction more explicit. A description of the temporal and spatial transformations of the postmitotic granule cells (*designated with numerals 1 through 7*) and stellate cells (*S*), as well as other details, are given in Rakic (1971b, 1973); *BGF*, Bergmann glial fiber; *CF*, climbing fiber; *D*, dividing external granule cell; *EG*, external granule layer; *GEC*, Golgi epithelial cell (Bergmann glia); *G*, granular layer; *M*, molecular layer; *MF*, mossy fibers; *P*, Purkinje layer; *PC*, Purkinje cell; *PCD*, Purkinje cell dendrite; *PF*, parallel fiber; S_{1-4}, stellate cells; *ST*, stellate cell dendrite. [Adapted from Rakic (1971b), with permission.]

Rakic, 1995; Rakic and Komuro, 1995). We found that blockade of N-type Ca^{2+} channels in cerebellar slices by using ω-conotoxin slows the rate of granule cell migration, although inhibitors of Cl^- and K^+ channels have no effect on the rate of granule cell migration (Komuro and Rakic, 1992). Likewise, application of *N*-methyl-D-aspartate (NMDA) receptor antagonists curtails cell migration, presumably by decreasing Ca^{2+} influx (Komuro and Rakic, 1993). The NMDA receptors may be activated by extracellular glutamate released mainly from ambient parallel fibers belonging to previously formed granule cells. However, neighboring glial cells may be involved in the regulation of glutamate. Indeed, inhibition of glutamate sequestration by the surrounding astroglia elevates endogenous extracellular glutamate and accelerates the granule cell migration rate (Komuro and Rakic, 1993). The molecular mechanisms of neuronal migration that involve participation of radial glial cells are among an active and promising area of research (e.g., see Rakic et al., 1994) and are not reviewed in detail here.

In addition to serving as a guide for migrating neurons and possibly for axonal growth cones (e.g., Silver et al., 1982; Norris and Kalil, 1991), the large surface of elongated glial fibers also may be involved in the signaling of information between proliferative zones and their distant terminations. Molecules, nutrients, and oxygen may be supplied by radial glial cells to the surrounding cells during embryonic stages when vascularization of the cerebral wall is absent or underdeveloped (Schmechel and Rakic, 1979a). The elongated fiber of radial glial cells may be involved in the transport process (Oksche, 1968) and would potentially enable them to communicate information from the developing cortical plate to centers of cell proliferation (Stensaas and Gilson, 1972). The possibility that such information is carried by molecules within the cytoplasm of radial fibers is supported by the demonstration of vigorous retrograde transport from endfeet to radial glial cell bodies (Ivy and Killacky, 1978). Modulation of glial cell behavior by the interaction of their distant processes has also been suggested to occur in newt optic tectum where deafferentiation of the molecular layer by enucleation of eyes resulted in a delayed increase in the mitotic activity of radial glial cells situated near the ventricular surface (Gaze and Watson, 1968). There is some evidence that the glial-limiting membrane formed by terminal attachments at the pial and vascular sheeting provides a modified blood-brain barrier and a cerebrospinal fluid brain barrier during embryonic stages (Oksche, 1968). Finally, the transient population of radial glial cells might also involve regulation of the voluminous extracellular fluid in loosely packed embryonic nervous tissue, a role postulated for astrocytes in adults (see Chapters 31, 47, and 48, this volume).

Disorders that are attributable to the damage of the transient glial scaffolding and indirectly to faulty neuronal migration range from gross malformations such as lisencephalia and polymicrogyria to the subtle heterotopia observed in the cerebrum of patients with developmental dyslexia and epilepsy. Because of multiple functions that involve interaction with the neighboring cells, and the transiency of radial glial cells, their involvement in the pathology is rather obscure, complex, and diverse. Usually, neuropathologists see the consequences long after the pathological reaction has been completed. Nevertheless, it is well recognized that the process of migration is highly sensitive to various physiological (e.g., ionizing radiation, heat), chemical (e.g., various drugs, carbon monoxide poisoning, excessive use of alcohol), and biological agents (some neurotropic viruses) as well as genetic mutations (e.g., Rakic and Sidman, 1973; Nowakowski, 1984; Caviness and Williams, 1985; Schull et al., 1986; Barth, 1987; Sherman et al., 1987; Caviness and Rakic, 1988). We only have begun to understand the role of radial glial cells in these defects. One scenario is that some of these agents interfere with neuron-glia interaction and indirectly impair neuronal pathfinding or cell motility. In many conditions, disruptive agents interrupt the radial glial fibers with little injury to neurons, which are, nevertheless, unable to move normally. For example, chronic exposure to alcohol may damage the radial glial cell membrane and interfere with the normal production and migration of neurons (Miller, 1986), and thus cause defects associated with fetal alcohol syndrome. Likewise, alcohol may affect the function of the NMDA receptor channel that regulates the rate of neuronal migration by controlling the influx of calcium ions (Komuro and Rakic, 1993). Malnutrition due to a low protein diet also affects development of glial scaffolding that may result in defects of neuronal migration. In spite of the relatively well-described pathological phenomena associated with defective neuronal migration, we are still ignorant about the cellular and molecular mechanisms underlying any of these processes.

DISSOLUTION AND EVOLUTION

In many submammalian species, radial glial cells persist throughout the life span (e.g., Ramón y Cajal, 1909; King, 1966). However, in primate telencephalon radial glial cells disappear, for the most part, at the end of neuronal cell migration to the cortex

(Rakic, 1972; Schmechel and Rakic, 1979a, 1979b). Analyses of transitional forms of radial glial cells in Golgi-stained and GFAP-immunolabeled sections prepared from cerebral cortex implied that radial glial cells were transformed into fibrillary astrocytes and/or protoplasmic astrocytes (Rakic, 1975, 1984; Choi and Lapham, 1978; Schmechel and Rakic, 1979a; Levitt and Rakic, 1980; Rickmann et al., 1987). The time table of the disappearance of radial glial cells in the primate neocortex, hippocampus, and cerebellum correlates with the initial appearance of astrocytes in these cortical regions (Rakic, 1971; Schmechel and Rakic, 1979a; Eckenhoff and Rakic, 1984).

A sequence of the morphogenetic transformations that lead to the formation of astrocytic cells in the adult neocortex, cerebellar cortex, and hippocampus are illustrated in Figure 49-8. Initially, the only glial cell elements present in all three regions are radial glial cells (Figure 49-8A–C). However, in the large primate telencephalon radial glial cell fibers attain enormous lengths, reaching several centimeters in the midterm human fetus (Rakic, 1984). In the macaque monkey, transitional forms between radial glial cells and astrocytes are first observed in the middle of the gestational period (Schmechel and Rakic, 1979b; Levitt and Rakic, 1980) a time period when the somata of some radial glial cells detach from the ventricular surface and become displaced to the intermediate zone (Figure 49-8). Subsequently, the cell forms of some radial glial cells may move to various depths of the cerebral wall and acquire typical, bushy astrocytelike appendages. Recently, the morphological transformation of radial glial cells into mature astrocytic cell classes has been confirmed following the uptake of fluorescent dyes by embryonic radial glial cells *in vivo* (Voigt, 1989).

The cellular or the molecular events that underlie the observed morphological transformation of radial glial cells are not known. In primary cultures of astrocytes, neuronal cells have been shown to exert an inhibitory effect on glial cell proliferation and, additionally, appear to regulate changes in astroglial cell shape from epitheliallike to radial or stellate (Sobue and Pleasure, 1984; Hatten, 1985, 1987; Ard and Bunge, 1988; Gasser and Hatten, 1990; Culican et al., 1990). Interestingly, in rhesus monkey a cessation in the mitotic activity of many radial glial cells accompanies the entire period of neuronal cell migration, which in this species approaches 2 months (Schmechel and Rakic, 1979b). Subsequent to the completion of neuronal cell migration, radial glial cells reenter the mitotic cycle, and many simultaneously undergo a transformation into astrocytes.

The morphological transformation of GFAP-positive radial glial cells into classical astrocytic cell forms, as well as alternative radial cell forms, appears to coincide with the loss of RC1, RC2, and Rat-401 antigens, which are not expressed in the adulthood (Hockfield and McKay, 1985; Misson et al., 1988a, 1988b; Edwards et al., 1990). A correlative analysis of antigenic and morphologic transformation *in vivo* (Caviness et al., 1989; Misson, 1991), *in vitro* (Culican et al., 1990) demonstrates that the morphological transformation of radial glial cells occurs concomitantly with a gradual acquisition of GFAP immunoreactivity and with a corresponding loss of RC1 immunoreactivity.

Many diverse mature forms of astrocytes, such as ependymoicytes, tanycytes, Bergmann glial cells, and Müller cells share basic morphological, immunological, and biochemical features with telencephalic radial glial cells (e.g., Bartlett et al., 1980; Edwards et al., 1990; Robinson and Dreher, 1990; for review see Fedoroff and Vernadakis, 1986 and Chapter 4). Therefore, these cells may be considered morphologically and biochemically divergent forms of embryonic radial glial cells that continue to be maintained in the adult mammalian brain. In the telencephalon, radial glial cells produce basically type-1 astrocytes. As suggested recently, a continued cell association with a selective microenvironment may allow for the maintenance of a modified radial glial cell form in the adult (Reichenbach, 1989). Based on the available data, it appears that both cell-cell interactions and cell lineages are likely to determine the fate of radial glial cells and their quantitative contribution to the astrocyte populations. Thus, the primordial fetal radial glial cell may be considered as a precursor that gives origin directly or indirectly to all major classes of astrocytes in the mammalian telencephalon (Rakic, 1984; Cameron and Rakic, 1991). While, during their short existence, radial glial cells contribute actively to brain construction, their progeny remain involved in brain functioning in health and disease during the entire life span.

As mammalian evolution proceeds from a smaller to a larger brain with an ever expanding surface of cerebral cortex, the radial glial cells increase in their number, their length, and the duration of their existence in individual embryonic development. Although the size of postmitotic cells does not increase appreciably from smaller to larger mammals, the journey that migrating neurons have to negotiate increases by an order of magnitude. In higher primates, including human, radial glial scaffolding is becoming more prominent than in any subprimate species (e.g., Figures 49-5 and 49-9). Based on a comparative embryological analysis of telencephalic development, we proposed the radial unit hypothesis

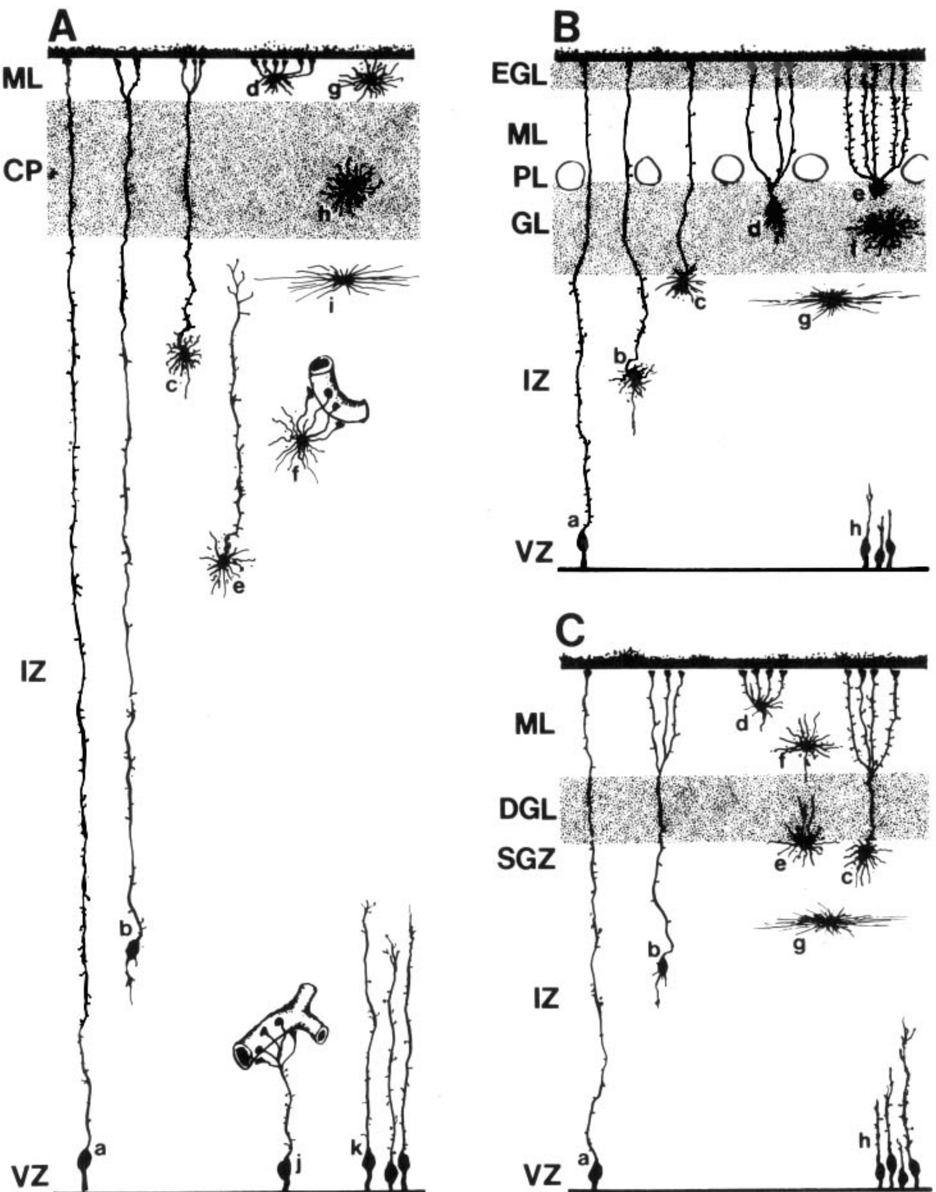


FIG. 49-9. Morphogenetic transformation of fetal radial glial cells into various astrocytic forms in the (**A**) cerebral hemisphere; (**B**) cerebellar hemisphere; (**C**) dentate gyrus of the hippocampus. *CP,* cerebral cortical plate; *EGL,* external granule layer; *GL,* granular layer (internal) of the cerebellum; *DGL,* dentate gyrus-granular layer of the hippocampal region; *IZ,* intermediate zone; *ML,* molecular layer; *PL,* Purkinje cell layer; *SGZ,* subgranular zone of the dentate gyrus; *VZ,* ventricular zone. Further explanation and designation of various transitional cell forms (*a–k*) is provided in the text. [From Rakic (1984), with permission.]

of cortical evolution (Rakic, 1990). According to this hypothesis, the mitotically active zone, situated at the surface of the cerebral ventricle, is depicted as a two-dimensional mosaic of proliferative units which constitute the rough protomap of the prospective species-specific cytoarchitectonic areas (Rakic, 1988b). Each radial unit consists of several clones that produce a cohort of neurons sharing a common site of origin in the ventricular zone, a common migratory pathway along the radial glial fascicle, across the intermediate and subplate zones, as well as the final columnar deployment (ontogenetic column) in the cortical plate (Figure 49-10). We postulated that the size of the cortical mantle, including the basic species-specific pattern of the cytoarchitectonic area, depends on the number of contributing radial units, while the thickness of the cortex depends on the magnitude of cell production within each unit. Although the initial number of units in a given species are likely to be set up early in embryogenesis by regulatory genes, the organization and final size of each cytoarchitectonic area is established

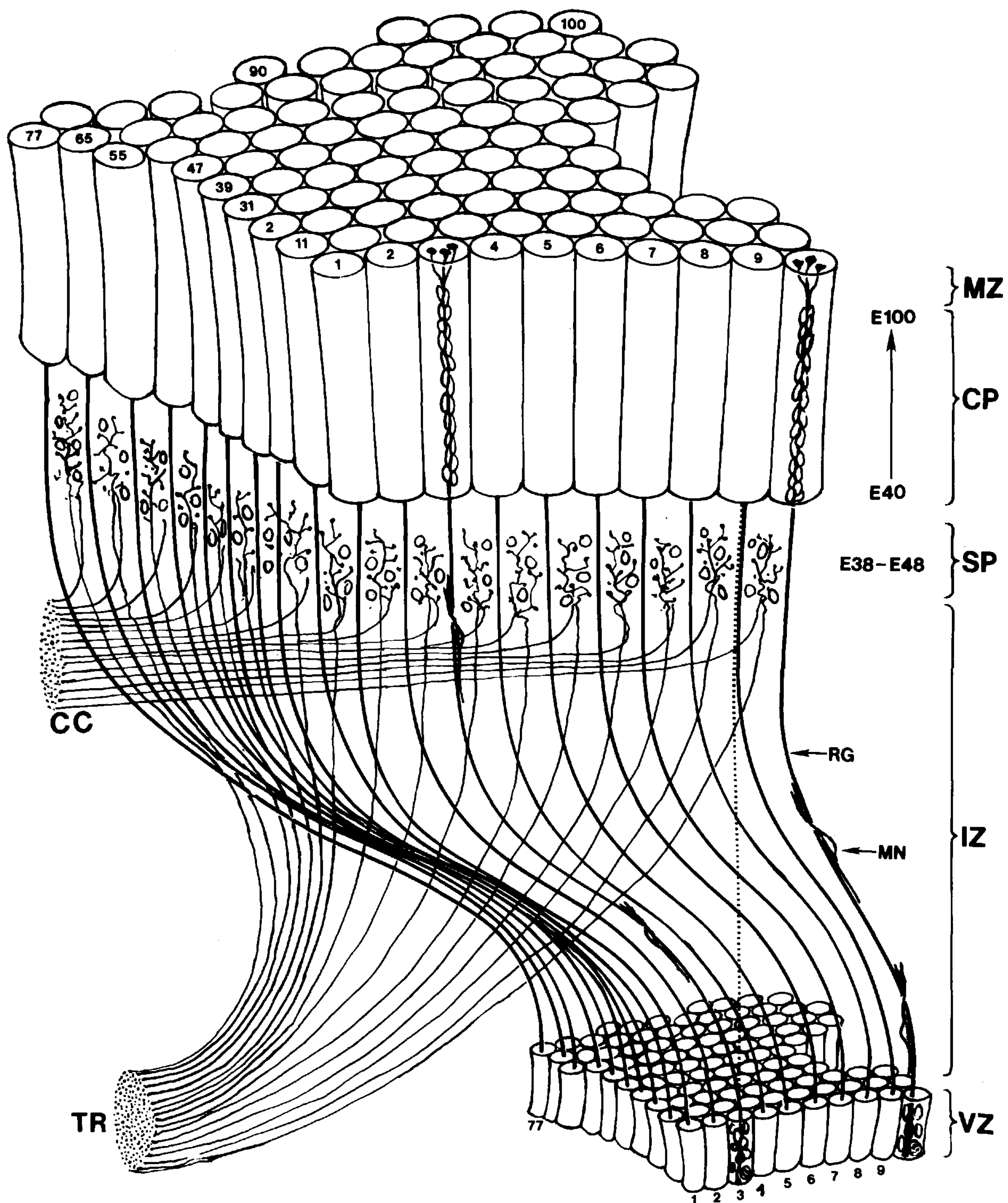


FIG. 49-10. The relationship between a small patch of the proliferative, ventricular zone (*VZ*) and its corresponding area within the cortical plate (*CP*) in the developing primate cerebrum. Although the cerebral surface in monkey expands during prenatal development, resulting in a shift between the *VZ* and *CP*, ontogenetic columns (*outlined by cylinders*) remain attached to the corresponding proliferative units by the grid of radial glial fibers. Cortical neurons produced by a given proliferative unit between embryonic (*E*) age 40 and E100 migrate in succession along common radial glial guides (*RG*) and form a single ontogenetic column. Each migrating neuron traverses the intermediate (*IZ*) and subplate (*SP*) zones, which contain "waiting" afferents originated in the thalamic radiation (*TR*) and other cortical areas in the same or other hemisphere (*CC*). After entering the cortical plate, each wave of migrating neurons bypasses the population of previously generated neurons and assumes a position at the interphase between the *CP* and marginal zone (*MZ*). As a result of this process, proliferative units 1–100 give rise to ontogenetic columns 1–100 that are arranged in the same relative position to each other. The glial scaffolding prevents a mismatch between proliferative unit 3 and ontogenetic column 9 (*dashed line*). Thus, the specifications of topography and/or modality depend on the spatial distribution of proliferative units, while the radial (*vertical*) position of neurons within each unit depends on its time of origin. [From Rakic (1988b), with permission.]

through interactions with appropriate afferents (Rakic, 1988b; Rakic et al., 1991). The enlargement of existing, or introduction of new, cytoarchitectonic areas in evolution could initially occur through a heterochronic process that includes a modification of the rate and cessation of cell proliferation during the phase of radial unit formation. We suggest that the new set of radial units interacts with afferent systems, creating an opportunity for the formation of novel input/target/output relationships, which, if heritable, may be subject to natural selection. Recent experiments on the embryonic brains of extant species, including induction of a novel cortical area by experimental manipulation of the developing thalamocortical input, support this hypothesis (Rakic et al., 1991). Thus, evolution of the neocortex may be intimately related to the evolution of radial glial scaffolding.

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50 Formation of the blood-brain barrier

HARTWIG WOLBURG AND WERNER RISAU

The blood-brain barrier represents the interface between the blood that supplies nutrients to the brain, and the brain that requires homeostasis of its environment to function properly. The concept of the blood-brain barrier developed over the last hundred years after Paul Ehrlich and his pupil Edwin E. Goldman injected dyes into the vascular system and the cerebrospinal fluid and observed staining of the whole organism except the brain or of the brain only and not the vascular system and the peripheral organs, respectively. The term "blood-brain barrier" was introduced by Lewandowski (1900) after Biedl and Kraus (1898) and he himself had performed experiments using neurotoxic agents that affected the brain only when injected directly into the brain and not when injected into a vein.

The barrier separates plasma from brain interstitial fluid. It includes the vascular-glial complex within brain tissue (endothelial blood-brain barrier) on the one side, and the choroid plexus epithelium, the tanycytes in the circumventricular organs, and the arachnoid barrier (blood-cerebrospinal fluid barrier) on the other (Figure 50-1). The circumventricular organs include the hypothalamic median eminence, the pituitary, choroid plexus, pineal gland, subfornical organ, subcommissural organ, organum vasculosum lamina terminalis, and the area postrema (Leonhardt, 1980). The direct exposure of neurons in these areas to the blood milieu is of fundamental importance. The choroid plexus, for example, is responsible for the formation of the cerebrospinal fluid and is in contact with bloodborne substances that penetrate through fenestrated capillaries. Since the cerebrospinal fluid itself has free access to the neurons through the ependymal cell layer (at least in higher vertebrates), a barrier is necessary to avoid the exchange of substances between blood and neuropil via the brain ventricle. This barrier is localized in the choroid plexus epithelium and called the blood-cerebrospinal fluid barrier. Correspondingly, the other circumventricular organs also require this type of barrier. For example, the median eminence contains neurons synthetizing glandotrope hormone-releasing factors, which freely enter the blood and are destined to activate hormone-producing cells in the distant adenohypophysis. This tolopogy is also the basis of important feedback control of neuronal activity within the neuroendocrine organs by "sensing" hormones and other messengers in the blood. The blood-cerebrospinal fluid barrier is formed here by specialized ependymal cells called tanycytes. Another barrier that protects the brain from the blood is at the border between the outer cerebrospinal fluid compartment and the leaky blood vessels of the dura mater. This barrier is formed by the neurothelium, which consists of flat cells of the arachnoid layer facing the dura mater. The main characteristic of all three subtypes of the plasma-brain interstitial fluid barrier (endothelium, tanycytes/choroid plexus epithelium, and neurothelium) is the extensive elaborate network of complex tight junctions (Nabeshima et al., 1975; van Deurs and Koehler, 1979; Nagy et al., 1984; Mollgard and Saunders, 1986).

The site of the blood-brain barrier was identified by Reese and Karnovsky (1967) and Brightman and Reese (1969) as the vascular endothelial cells. This applies to all vertebrates except elasmobranch fishes, which have a glial blood-brain barrier (Cserr and Bundgaard, 1984) as do many invertebrates (Abbott, 1991; Lane, 1991). Generally, all blood vessels of the organism are lined by endothelial cells; abluminally they are associated with pericytes and/or smooth muscle cells. In peripheral organs, blood vessels allow more or less free access of bloodborne substances to the subendothelial space; access is provided by endothelial junctions, fenestrations, or discontinuities in the capillary walls, whose endothelial cells additionally are rich in pinocytotic vesicles. The tight junctions of brain endothelial cells alone form the structural basis of the barrier. A further prerequisite of impermeability is a low pinocytotic activity across the barrier cells (Peters et al., 1991), although a unidirectional transcytosis of proteins from blood to brain was recently described (Villegas and Broadwell, 1993).

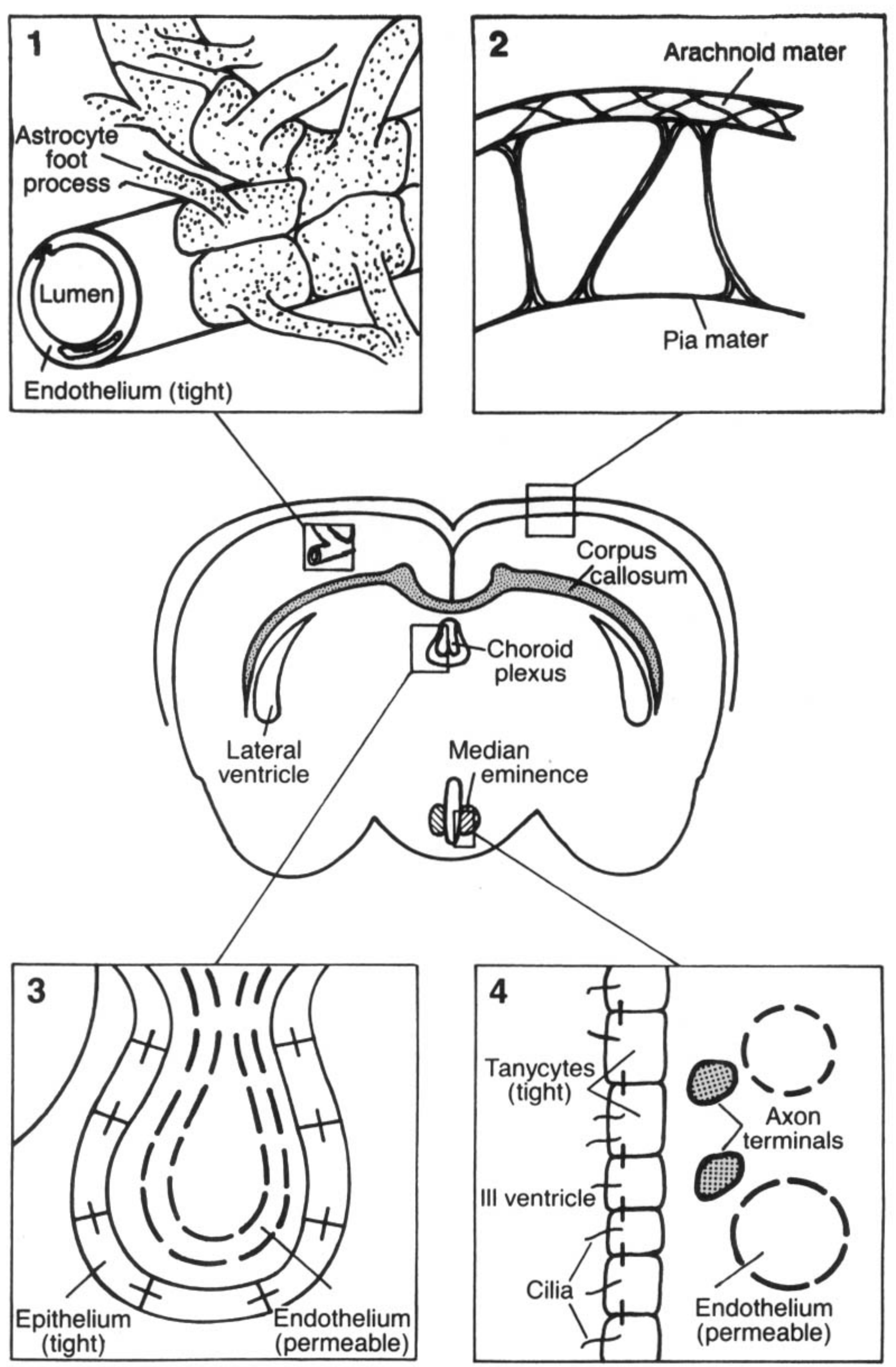

FIG. 50-1. Barriers and compartments in the brain. **Center:** Frontal section of a rat brain in the region of the median eminence. **Inset 1:** Endothelial blood-brain barrier. **Insets 2–4:** Blood-cerebrospinal fluid barrier—*inset 2:* the meningeal barrier at the surface of the brain; *inset 3:* the choroid plexus, which produces the cerebrospinal fluid out of bloodborne substances penetrating through fenestrated endothelia; *inset 4:* the blood-cerebrospinal fluid barrier in circumventricular organs such as the median eminence where tight junction-connected tanycytes avoid the exchange of substances between the ventricle and the neuropil. [From Risau and Wolburg (1990), with permission.]

DIFFERENTIATED PROPERTIES OF VERTEBRATE BRAIN ENDOTHELIAL CELLS

The fact that tight junctions occlude the intercellular cleft between endothelial cells and pinocytotic activity is reduced in the endothelium demands the existence of specific carrier systems for the transport of essential substrates of energy and amino acid metabolism to the brain. Hydrophilic substances such as proteins, amino acids, carbohydrates, and ions are normally not able to diffuse through endothelial membranes. Their transport is dependent on the availability of special transport systems or receptors. In fact, several specific carrier systems in brain endothelial cells were identified that allow the regulated exchange of substances between blood and brain. As a carrier of outstanding importance for the energetic supply of the brain, the glucose transporter was found to be inserted in high concentration in both the luminal and the abluminal membrane of brain capillary endothelial cell (for review see Dermietzel and Krause, 1991). This erythrocyte-type, insulin-independent, glucose transporter (GluT-1) is slightly asymmetrically expressed in the endothelium, in that more transporters appear to be inserted in the abluminal than in the luminal membrane (Farrell and Pardridge, 1991).

Other transporters found in the brain capillary endothelium are specific for amino acids (reviewed in Dermietzel and Krause, 1991) (Figure 50-2). The L-system is responsible for the transport of large neutral amino acids such as leucine, isoleucine, phenylalanine, dihydroxyphenylalanine (DOPA), valine, and tryptophan and is localized in both luminal and abluminal membranes of endothelial cells (Betz and Goldstein, 1978). Dopamine can penetrate the endothelium only through the abluminal membrane. Therefore, patients with Parkinson's disease who have serious dopamine deficits in the brain, cannot be treated by dopamine administration; however, treatment with L-DOPA is of limited efficiency as well as it enters the endothelium via the L-system and is converted into dopamine and DOPAC by the endothelial enzymes DOPA-decarboxylase and monoamine oxidase, respectively ("metabolic" blood-brain barrier; Goldstein and Betz, 1986). The so-called A-system, which is restricted to the abluminal membrane of endothelial cells selects small neutral amino acids such as glycine, alanine, and γ-aminobutyric acid (GABA) and cotransports Na^+-ions into the endothelial cytoplasm. In the luminal membrane, Na^+-influx and a Na^+/Cl^--cotransporter has been demonstrated (Betz, 1983). Na^+ would activate the abluminal Na^+-K^+-ATPase which in turn increases the uptake of K^+ from the brain interstitium into the blood through luminal K^+-channels. This could be facilitated by the activation of abluminal stretch-activated inward-rectifying Na^+/Ca^{2+}-channels (Popp et al., 1992) (Figure 50-2).

Another system possibly involved in the amino acid transfer from blood to brain and vice versa may be gamma-glutamyl transpeptidase (GGT). This enzyme catalyzes the transfer of gamma-glutamyl residue of glutathione to amino acids (Orlowski and Meister, 1970). Commonly, it was held to be a specific marker of brain capillary endothelium. However, new results have shown that this transpeptidase activity is associated with pericytes rather than with endothelial cells (Frey et al., 1991; Risau et al., 1992). Moreover, the finding that GGT is also present in permeable blood vessels speaks against the suggestion that this enzyme might be a reliable marker of blood-brain barrier (Risau, 1991). The role of GGT and other peptidases at the blood-brain barrier was comprehensively discussed recently by Brownlees and Williams (1993).

The impermeability of the paracellular pathway in brain endothelium requires—besides the facilitated diffusion of sugars and amino acids by appropriate transporters—mechanisms specific for the uptake of proteins such as transferrin, immunoglobulins, insulin, and other peptides and hormones (for reviews see Dermietzel and Krause, 1991). Evidence for the presence of coated pits (Broadwell, 1989), adsorptive transcytosis of proteins (Villegas and Broadwell, 1993), and receptors for transferrin, insulin, and low-density lipoprotein (LDL) (Jefferies et al., 1984; Duffy and Pardridge, 1987; Méresse et al., 1989a) in blood-brain barrier endothelium has been presented (see Figure 50-2). Receptor-mediated endocytosis of transferrin at the blood-brain barrier has been conclusively demonstrated by Roberts et al. (1993). However, the mechanism by which iron dissociates from the transferrin within the endothelium and is released from the abluminal pole of the vascular wall into the brain is unknown so far. Transferrin including its mRNA is present at least in oligodendrocytes, but there is no information available on the balance between intrinsic brain transferrin and transferrin derived from the blood (for further discussion see Roberts et al., 1993).

All transport systems described so far concerned hydrophilic compounds, which require special mechanisms of facilitated diffusion or specialized transport mechanisms. Unrestricted diffusion through the blood-brain barrier is, in principle, possible for substances that can cross biological membranes by virtue of their lipophilic properties. For these substances, control mechanisms might also exist that actively export them as soon as they have entered the brain en-

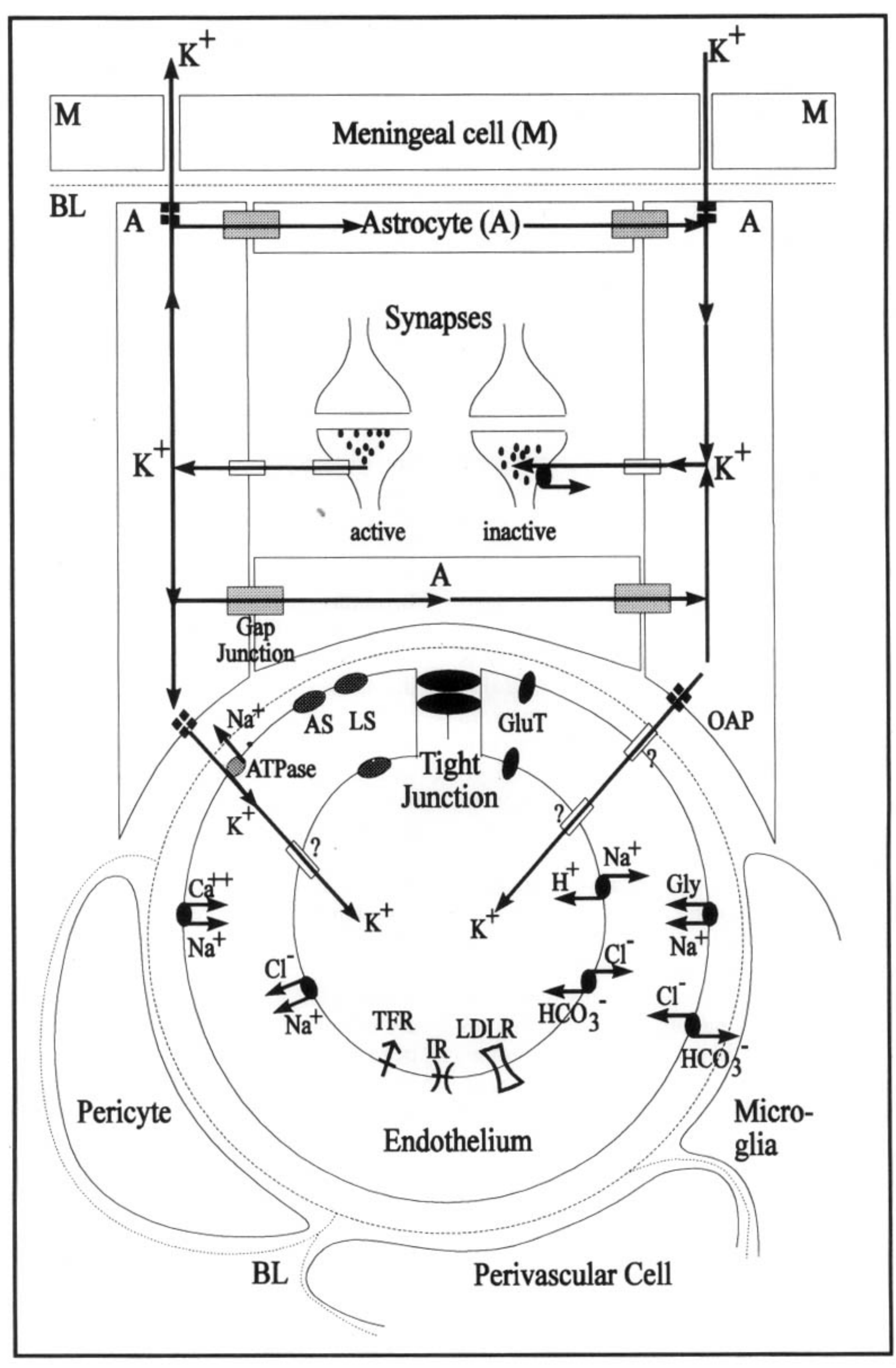

FIG. 50-2. Schematic and hypothetical representation of some intercellular (glioneuronal and gliovascular) communications in the central nervous system. The upper part of the figure shows the astrocytic syncytium, which is involved in K^+ homeostasis. Astrocytic endfeet, together with other cell types or their processes (microglial cells, perivascular cells, and pericytes) contact the perivascular basal lamina. In the endothelium, some of the known transporters, channels, and receptors are schematically illustrated. *AS*, A-system of amino acid transport (alanine preferring); *BL*, basal lamina; *GluT*, glucose transporter; *IR*, insulin receptor; *LDLR* low density lipoprotein receptor; *LS*, L-system of the amino acid transport (leucine preferring); *OAP*, orthogonal arrays of particles; *TFR*, transferrin receptor. [Modified from Risau and Wolburg (1990) and Risau (1991), with permission.]

dothelium. For example, the P-glycoprotein is a transmembrane glycoprotein that actively extrudes nonpolar molecules out of endothelial cells. It confers insensitivity for drugs for tumor chemotherapy (multidrug resistance) to cells that express this transporter, and has been shown recently to be expressed in blood-brain barrier endothelium (Cordon-Cardo et al., 1989). For example, vincristine applied to the vasculature was shown to be concentrated in the cerebrospinal fluid at a considerably lower level as compared to simultaneous plasma levels demonstrating a restricted entry through the blood-brain barrier endothelium (Jackson et al., 1981).

Several proteins have been localized to brain capillaries *in vivo* [e.g., alkaline phosphatase (Leduc and Wislocki, 1952), butyryl-cholinesterase (Joo and Csillik, 1966)]. By analogy to other cell types that express these proteins, they have been implicated in transport or metabolic processes at the blood-brain barrier. However, direct evidence for such a role is lacking. Recently, cytokines such as tumor necrosis factor were found to influence the blood-brain barrier permeability; however, the mechanism of how these mediators might operate in the brain endothelial cells is still a matter of speculation (Megyeri et al., 1992). Also, nitric oxide (NO) as an activator of the guanylate cyclase may play a role in the gliovascular interrelationship (Murphy et al., 1993). In the peripheral circulation, NO has been characterized as a potent relaxing factor. In brain microvessel endothelial cells the cytokine-inducable NO synthase has been detected by Durieu-Trautmann et al. (1993). Astrocytes as well as microglial cells are thought to be target cells of neuronal and endothelial NO; in addition, astrocytes and microglial cells have been shown to display NO synthase activity (for review see Murphy et al., 1993). Direct functional implications of the NO system for the regulation of the blood-brain barrier are not known so far. Furthermore, proteins have been identified in brain capillaries using monoclonal antibodies (Michalak et al., 1986; Risau et al., 1986b; Sternberger and Sternberger, 1987). Some of these proteins may be blood-brain barrier-specific in that sense that they are present at a much reduced or undetected level in permeable capillaries of the body. Some proteins present in other endothelial cells seem to be absent from brain endothelium. For example, the acetylated low-density lipoprotein (acLDL) receptor (Gaffney et al., 1985), thrombomodulin (Ishii et al., 1986), and the OX43 (Robinson et al., 1986) antigen seem to be downregulated in brain endothelial cells.

The HT7 protein is a cell surface immunoglobulinlike glycoprotein and a marker for blood-brain barrier endothelium (Risau et al., 1986a, 1986b; Albrecht et al., 1990; Seulberger et al., 1990). It is expressed on the surface of chick brain endothelial cells *in vivo* and *in vitro*. Recently, the OX-47 (Fossum et al., 1991) and gp42 (Altruda et al., 1989) proteins have been identified as homologous proteins in rat and mouse, respectively. We found that the OX-47 protein was present in rat brain endothelial cells throughout the culture period in primary and passaged cells suggesting that this protein is not downregulated *in vitro* (Risau, 1991). This is consistent with our results using HT7 antibodies in the chick (HT7 is a species-specific antibody). There is compelling evidence that the expression of this protein is modulated *in situ* and *in vitro* (Lobrinus et al., 1992). It is present in erythroblasts but not erythrocytes and it is induced in endothelial cells that invaded a brain transplant (Risau et al., 1986a). In addition, the 1W5 protein or neurothelin (Schlosshauer and Herzog, 1990), which is identical to the HT7 protein (Seulberger et al., 1992) is induced in neurons *in vitro*, and the OX-47 protein is expressed by activated but not resting T lymphocytes (Fossum et al., 1991). These results raise the possibility that if downregulation of this protein occurs by brain endothelial cells during the time in culture this may be masked by its simultaneous induction by the culture conditions. Thus, at this point it is impossible to distinguish between maintenance of protein expression due to maintenance of differentiated characteristics of brain endothelial cells and induction due to culture conditions. Studies are in progress to address this issue.

MORPHOLOGICAL PROPERTIES OF VERTEBRATE BRAIN MICROVESSELS AND ASSOCIATED CELLS

The morphology of brain microcirculation is well known and amply described (e.g., Wolff, 1963, 1987; Reese and Karnovsky, 1967; Brightman and Reese, 1969; Bär, 1980; Bradbury, 1984; Cserr and Bundgaard, 1984; Nagy et al., 1984; Coomber and Stewart, 1985; Mollgard and Saunders, 1986; Lane, 1991; Peters et al., 1991). In contrast to blood vessels outside the central nervous system, the brain capillaries show endothelia that are seamless or connected by complex tight junctions (Figures 50-3, 50-4, and 50-5). These tight junctions display impermeability of electron dense tracers such as horseradish peroxidase (MW 40,000), microperoxidase (MW 800), lanthanum hydroxide or thorium dioxide. Vessels in the circumventricular organs contain fenestrated endothelial cells and are leaky for these tracers (Dermietzel and Leibstein, 1978; Peters et al., 1991). Additionally, the perivascular space

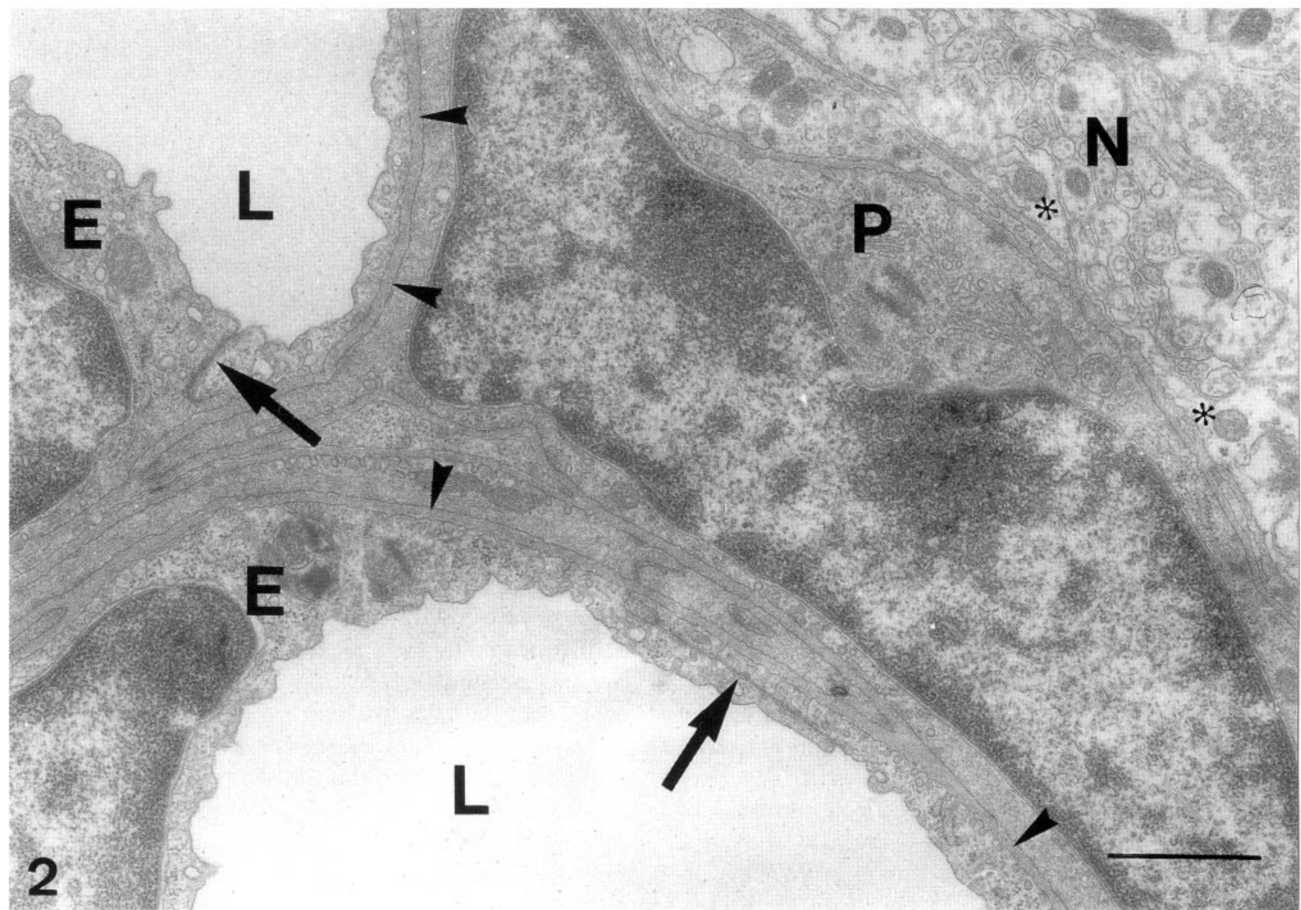

FIG. 50-3. Ultrathin section through small arterioles in the mouse cerebral cortex. *L,* vessel lumen; *E,* endothelial cell; *P,* pericyte or smooth muscle cell; *N,* neuropil. *Arrows,* endothelial tight junctions; *arrowheads* the thick basal laminae; *asterisks,* astrocytic endfeet. Bar = 1 μm.

around leaky vessels is large and filled with extracellular matrix (Dermietzel and Krause, 1991). In these vessels, the basal lamina is split into a subendothelial one and an astroglial one. During development of blood-brain barrier vessels, the perivascular space becomes smaller and smaller (Caley and Maxwell, 1970). The perivascular space of tight brain capillaries is very narrow and occupied by a common basal lamina that covers both the basal membranes of endothelial cells and the perivascular astroglial endfeet. In cerebral arterioles, pericytes, perivascular cells, and smooth muscle cells are completely surrounded by their own basal lamina (Graeber et al., 1992). Smooth muscle cells and pericytes are hardly distinguishable by ultrastructural criteria (Figure 50-3). Perivascular microglial cells are covered by a basal lamina only where they contact the endothelium (Lassmann et al., 1991). The astrocytes regularly ensheathe the blood vessels (Wolff, 1963; Brightman and Reese, 1969; Goldstein and Betz, 1986; Peters et al., 1991). Each astrocyte projects only one main process to a given capillary, otherwise giving rise to en passent contacts with the capillary wall (Wolff, 1987). Astroglial gap junctions are preferentially found between perivascular endfeet or processes.

In the freeze-fracture replica of astroglial endfoot membrane contacting the subendothelial basal lamina, orthogonal arrays or assemblies of particles (OAP) are visible (Landis and Reese, 1981) (Figure 50-5). These particles whose subunits measure 6 to 7 nm in diameter are believed to be transmembrane proteins (Landis and Reese, 1989) and develop perinatally in astroglial membranes (Anders and Brightman, 1979; Landis and Reese, 1981). They form clusters of 4 to more than 30 subunits covering each about 1000 nm^2 and about 50% of the endfoot membrane area. However, in areas where the endfoot looses contact with the basal lamina by deflection of the membrane towards the neuropil, the OAP density drops to 40 to $50/\mu^2$, corresponding to 4 to 5% of the membrane area in the neuropil. The OAP

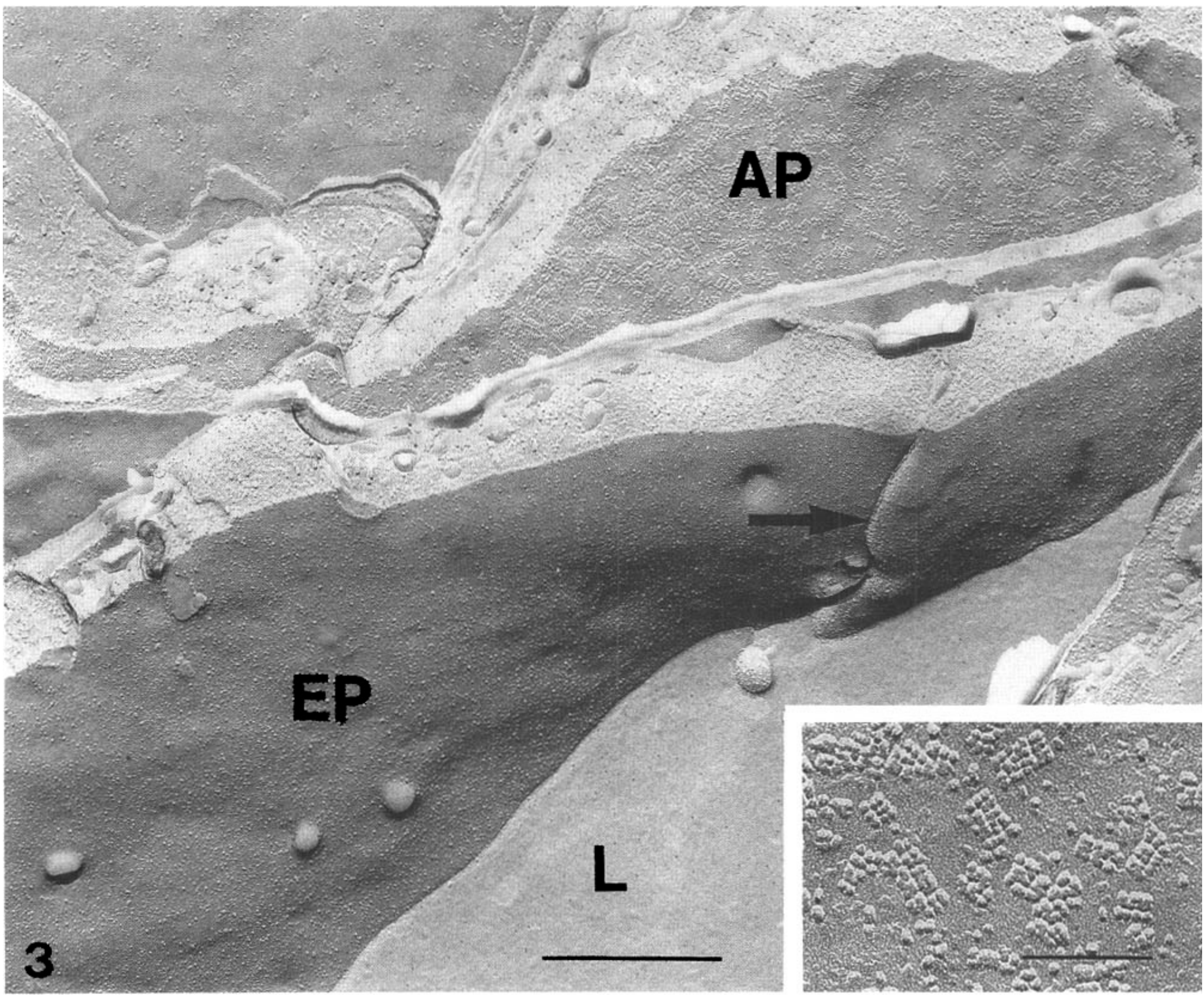

FIG. 50-4. Freeze-fracture replica of a capillary in the rat optic nerve. *L*, vessel lumen; *EP*, protoplasmic fracture face (P-face) of the luminal membrane of the endothelial cell; *AP*, P-face of an astrocytic endfoot with orthogonal arrays of particles (*higher magnification in the insert*); *arrow*, an endothelial tight junction (*membrane plane not exposed*). Bar = 0.5 μm (*insert:* bar = 0.1 μm).

appear to be of vital significance for the working central nervous system as was suggested by (1) the absence of OAP in Müller cells *in vitro* in chicken retinae where morphogenesis took place, but physiological activity was not demonstrable (Wolburg et al., 1991), and (2) by the formation of OAP in Müller cells being dependent on retina maturation in chicken and pigeon (Bolz and Wolburg, 1992). These data suggest that neuronal activity as well as the contact with the basal lamina seem to be synergistically required to produce an OAP-related glial polarity (for further discussion see also Wolburg and Bäuerle, 1993). On the other hand, the formation of blood-brain barrier begins early in development [E13 in chicken (Wakai and Hirokawa, 1978), E14 in mouse (Risau et al., 1986a), for example] suggesting that neuronal migration and synaptogenesis requires a strict control of the microenvironment as well (Mollgard and Saunders, 1986). However, the definitive formation and maturation of the blood-brain barrier takes time up to postnatal age (Stewart and Hayakawa, 1987; Risau and Wolburg, 1990). In the differentiated brain, neuronal activity leads to extracellular increase in [K^+], which can be balanced better in more advanced stages of brain development (Connors et al., 1982) (see Figure 50-2). Presumably, the capability of the brain to stabilize extracellular ion concentrations is coupled to maturation of glia (Laming, 1988), including the polar distribution of OAP in astrocytic membranes. Astrocytes are believed to be the main site of spatial buffering due to their polarized conductance for K^+ ions. Thus, glial

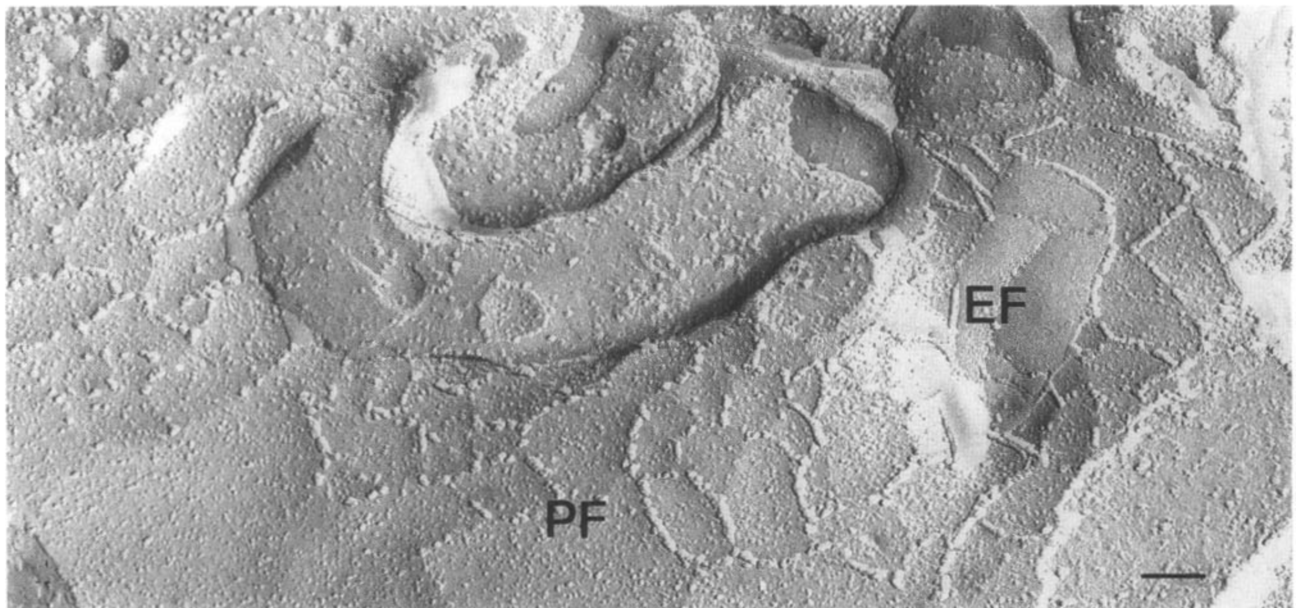

FIG. 50-5. Freeze-fracture replica of a capillary fragment freshly isolated from bovine brain. Tight junctions are associated with the P-face (*PF*); at the E-face (*EF*) grooves are sparsely occupied by single particles. Bar = 0.1 μm.

endfeet will shift K^+ from sites of high neuronal activity to perivascular sites where the K^+ concentration is maximal. The presumably high subendothelial K^+ concentration may be taken up by the abluminal Na^+/K^+-ATPase of the endothelium and released into the blood through luminal K^+-channels which are activated by intracellular Ca^{2+} (Popp et al., 1992) (Figure 50-2).

The lamina cribrosa of the optic nerve is a site where the blood-brain barrier is leaky (Tso et al., 1975; Flage, 1977). This fact was discussed regarding possible influences of plasma substances getting free access through leaky choroid vessels onto the marginal glial endfeet modulating certain properties of astrocytes within this region (Perry and Lund, 1990; Wolburg and Bäuerle, 1993). In most mammals, except of the rabbit, the lamina cribrosa is unmyelinated, giving rise to the hypothesis that it may be a barrier for migration of oligodendrocytes into the retina (ffrench-Constant et al., 1988). Interestingly, in the optic nerve of a myelin-deficient rat mutant (Rohlmann et al., 1992) as well as in the lamina cribrosa of the otherwise myelinated rat (Wolburg and Bäuerle, 1993) the density of OAP in astroglial endfeet is reduced. It seems therefore that where axons are nonmyelinated (including some of the circumventricular organs and the olfactory system) the blood-brain barrier is leaky (Perry and Lund, 1990) and astrocytes are altered (see also Dermietzel and Leibstein, 1978). Conversely, endothelial cells in culture are able to influence the OAP density in cocultured astroglial cells inducing tightly packed OAP (Tao-Cheng et al., 1990). Although the causal relationships between astrocytes, oligodendrocytes, and endothelial cells are far from being understood, the observations suggest that a high density of OAP in perivascular astrocytic endfeet belong to the set of characteristics associated—in higher vertebrates—specifically with the establishment of a functional blood-brain barrier.

ENDOTHELIAL TIGHT JUNCTIONS

Tight junctions are contact zones between epithelial cells that restrict or prevent the paracellular pathway ("gate" function as attributed by Mandel et al., 1993; for review see Madara and Pappenheimer, 1987). They form networks of strands in both partner cell membranes, which are believed to occlude the intercellular cleft. A close correlation between the complexity of these strands and the transepithelial electrical resistance has been demonstrated (Claude and Goodenough, 1973; Claude, 1978). They also contribute to the polarity of epithelia in that apical membranes differ from basolateral membranes in terms of lipid composition ("fence" function as attributed by Mandel et al., 1993). Many models were proposed to describe the molecular fine structure of tight junctions (for recent review see, for example, Lane et al., 1992); nevertheless, their biochemical identity is unknown as yet. Only associated

proteins such as the ZO-1 protein and cingulin were described (Stevenson et al., 1986; Citi et al., 1988). ZO-1 is a monoclonal antibody against a 225 kD protein associated with the cytoplasmic portion of tight junctions. The protein is present in brain capillaries *in vivo* (Watson et al., 1991) and in cultured endothelial cells (Rubin et al., 1991). Stevenson et al. (1988) have shown that the amount of ZO-1 protein did not correlate with junctional complexity and electrical resistance in MDCK cells. They suggested that the phosphorylation state of this protein might be important for the determination of the "tightness" of junctions. Thus, the regulation of protein phosphorylation in brain endothelial cells may be of major importance for the regulation of blood-brain barrier properties. These observations are consistent with results on brain endothelial cells (Risau, 1991), suggesting that the mere presence of the ZO-1 protein defined by the monoclonal antibodies is not diagnostic for blood-brain barrier properties of endothelial cells.

The electrical resistance of brain microvessels is established during development at a later stage than the impermeability for electron-dense tracers (for review see Rubin, 1991). Butt et al. (1990) reported impermeability for lanthanum in pial vessels of rat embryos, but an electrical resistance of only 300 ohm cm^2. Subsequently, the resistance increased up to greater than 1000 ohm cm^2. Provided that this is also valid for intracerebral microvessels (see Crone and Olesen, 1982) it could be suggested that a fully differentiated gliovascular system might be a prerequisite for the final maturation and maintenance of the blood-brain barrier. Detailed studies on the correlation between electrical resistance and tight junction structure in blood-brain barrier endothelial cells *in vivo* do not exist as yet. Instead, some related work has been performed in epithelial cells. Stevenson et al. (1988) have described low and high resistance MDCK cell tight junctions to be structurally identical. However, their pictures reveal that in low resistance MDCK cells more tight junction particles are associated with the E-face than in high resistance MDCK cells. In most epithelial tight junctions, such as in liver, intestine, kidney, or the retinal pigment epithelium, the tight junction strands are associated with the P-face; at the E-face, particle-free grooves predominate. Few E-face associated particles correspond with sites of interruptions of P-face associated strands as was shown in complementary freeze-fracture replicas (Noske and Hirsch, 1986; Mandel et al., 1993). In the vascular system, the tight junctions vary not only in terms of complexity of the strand network but also in terms of their association with the two membrane leaflets when regarding different segments of the vasculature. For example, venous as well as arterial endothelium show a poorly anastomosed network, with largely discontinuous strands associated with the E-face. At the P-face only particle-free ridges are seen (Simionescu et al., 1976; Mühleisen et al., 1989). Brain endothelial tight junctions are strongly anastomosed and associated with the P-face (Nagy et al., 1984; Shivers et al., 1984), although not so pronounced as in epithelia (Figure 50-5); for direct comparison of the tight junction structure in leaky endothelium and in tight pigment epithelium see Kniesel and Wolburg, 1993). Zampighi et al. (1991) and Mandel et al. (1993) have shown that the association of the tight junctions with one or the other leaflet of the membrane correlates with the permeability of the epithelial monolayer. Energy depletion results in both abolishing the "gate" function (increase of permeability) and inversion of the fracture pattern (increase of the number of E-face associated TJ-particles).

ASTROCYTES INDUCE THE BLOOD-BRAIN BARRIER *IN VIVO*

An important question is whether the blood-brain barrier properties of the endothelial cells are predetermined during early development of the vascular system or are induced after invading the brain environment. Several studies are in favour of the latter possibility. When transplanted into ectopic sites of host embryos of a different species, embryonic brain tissue has been shown to induce both angiogenesis in the host and blood-brain barrier characteristics in the invaded blood vessels. Conversely, when peripheral tissue was transplanted into the brain the invading capillaries lost their blood-brain barrier properties (Stewart and Wiley, 1981; Risau et al., 1986a). From these studies it was concluded that it is the brain microenvironment that determines the blood-brain barrier characteristics such as tight junctions of high complexity, high electrical resistance, and low permeability for lipid-insoluble substances. The early suggestion that astrocytes might play a role in this induction mechanism was substantiated by experiments performed by Janzer and Raff (1987). They provided direct evidence that astrocytes transplanted into the rat anterior eye chamber were capable of inducing a permeability barrier for dyes administered to the vascular system.

BLOOD-BRAIN BARRIER *IN VITRO*

The blood-brain barrier is a complex structure comprised of different cell types. In order to study in

more detail the role of single components of the blood-brain barrier, many efforts have been made to establish an *in vitro* blood-brain barrier system (Goldstein et al., 1984; Abbott et al., 1992; for review of the literature see Rubin, 1991; Joo, 1993). Pure endothelial cells have been isolated and examined as to whether or not the blood-brain barrier is maintained. Several lines of evidence suggest that cultured brain endothelial cells rapidly loose blood-brain barrier characteristics therefore not providing a suitable model of blood-brain barrier studies *in vitro*. On the other hand, many reports in recent years support the early suggestion that cocultured astrocytes or glioma cells may be able to reinduce blood-brain barrier properties in cultured endothelial cells. These properties included the activity of γ-glutamyl transpeptidase (De Bault and Cancilla 1980; De Bault, 1981; Maxwell et al., 1987; Dehouck et al., 1990; Tontsch and Bauer, 1991), the transport of neutral amino acids (Cancilla and De Bault, 1983), the polarity and activity of Na^+-K^+-ATPase (Beck et al., 1986; Tontsch and Bauer, 1991), the glucose uptake (Maxwell et al., 1989), and the expression of the HT7 protein (Lobrinus et al., 1992).

Of special interest is the question whether also the high electrical resistance and the complex tight junctions are among the properties of endothelial cells which can be reinduced and maintained in culture by astrocytes or other cells and factors. Indeed, Arthur et al. (1987) and Shivers et al. (1988) provided evidence for an astrocyte-mediated induction of tight junctions in cell culture which was also seen using astrocyte conditioned medium. Tao-Cheng et al. (1987) and Tao-Cheng and Brightman (1988) were also able to induce tight junctions in endothelial cells by astroglial cells *in vitro*, but their studies suggested that cell contact is required and that released factors are not sufficient. Neuhaus et al. (1991) have shown in a transfilter coculture system with bovine brain endothelial cells and rat astroglial cells cocultured separately on both sides of a cellagen filter which did not allow the direct contact of cell processes that complex tight junctions can be reinduced by astrocytes. Rubin et al. (1991) have shown that astrocyte-conditioned medium as well as increased cAMP levels enhanced both anti-ZO-1 immunoreactivity and the transendothelial electrical resistance in cultured bovine brain endothelial cells. Since ZO-1-immunoreactivity cannot be considered as a reliable marker of tight junction complexity, and since the absolute values of electrical resistance observed in endothelial cells are essentially lower than those measured in intact blood-brain barrier vessels (Butt et al., 1990; Crone and Olesen, 1982; Rutten et al., 1987), it may be concluded that culture conditions are hitherto not sufficient to mimick blood-brain barrier properties completely. It is of considerable interest that all published freeze-fracture replicas of cultured endothelial cells (Goldstein et al., 1984; Shivers et al., 1985, 1988; Arthur et al., 1987; Tao-Cheng et al., 1987; Méresse et al., 1989b) reveal tight junctions predominantly associated with the E-face (Figure 50-6). In contrast, high resistance MDCK cells or pigment epithelial

FIG. 50-6. Freeze-fracture replica of cultured bovine brain endothelial cells. The tight junctions are predominantly associated with the E-face (*EF*). *Arrows* mark ridges at the P-face (*PF*), which possess essentially fewer particles than that of freshly isolated endothelial cells (Figure 50-4). Bar = 0.1 μm.

cells do not loose the P-face association of tight junction particles *in vitro,* unless they suffer from energy depletion (Mandel et al., 1993). It appears that endothelial differ from epithelial tight junctions in that they require a constant impulse to maintain their tight junction integrity which may be lost due to a decay of tight junction-cytoskeleton connections (for further literature see Hageman and Kelly, 1985; Lane et al., 1992). This hypothesis would explain (1) the fact that blood-brain barrier endothelial cell tight junctions *in vivo* differ from those *in vitro* by the switch of the tight junction strands from the P- to the E-face, (2) the lower electrical resistance in cultured endothelial cells in comparison to intact blood-brain barrier capillaries, and (3) the sensitivity of brain capillaries against pathological disorders such as tumors, Alzheimer's disease, multiple sclerosis, and AIDS-associated syndromes. Apparently, brain endothelial cells need constant input from the brain microenvironment to maintain a functional blood-brain barrier. The most important goal in future research is the molecular identification of these factors.

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51 Schwann cell functions in saltatory conduction

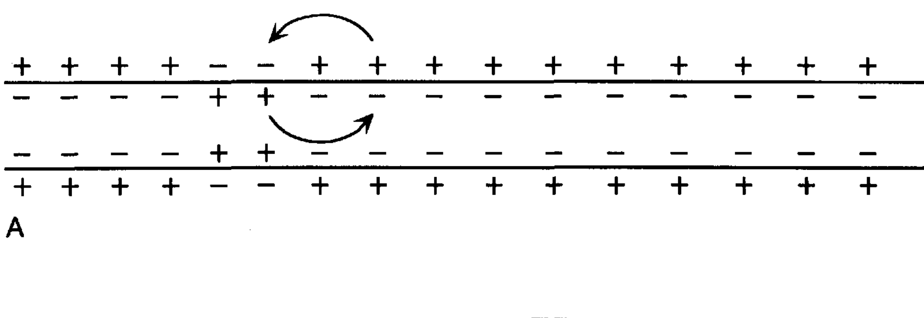
S. Y. CHIU

Schwann cells provide the myelin sheath for large axons of the peripheral nervous system, insulating regions of axons called the internode that are typically 1000 to 2000 μm long in large fibers. The axon is exposed between successive internodes at tiny gaps, each about 0.5 to 1.0 μm wide, called nodes of Ranvier. In the early 1950s, Stampfli, Huxley, and Tasaki provided the crucial piece of evidence that the node of Ranvier is the site of nerve excitation, with each node generating a high density of inward sodium current that helps excite consecutive nodes, leading to a mode of conduction termed saltatory conduction. The purpose of this chapter is twofold. First, it describes the classical role of Schwann cells in insulation and how they allow fibers to conduct with speed and efficiency. The treatment will be brief because this classical function of Schwann cell has been extensively reviewed (Hodgkin, 1964; Hille, 1977; Waxman, 1980). Second, it deals extensively with novel but somewhat more speculative roles of Schwann cells in relation to axonal functions based on recent discoveries that these cells express voltage-sensitive ion channels.

CLASSICAL ROLE OF SCHWANN CELLS IN NERVE CONDUCTION

Figure 51-1 summarizes the classical role of Schwann cells as providers of myelin and the two major advantages myelinated fibers have over nonmyelinated fibers of comparable size: faster conduction and lower energy expenditure. How myelination increases conduction velocity is best examined by first considering the mode of conduction of a nonmyelinated fiber (Figure 51-1A). Excitation is initiated at a small membrane area by membrane depolarization. This passive membrane depolarization leads to an activation of sodium channels, causing a regenerative influx of positive charges. This generates a local current that flows to depolarize the neighboring quiescent patch and triggers, in turn, a wave of sodium channel excitation that propels an undiminished wave of depolarization along the fiber at a constant velocity. In the case of nonmyelinated axons, the conduction is more or less continuous as the impulse propagates along excitable membrane patches that are immediately adjacent to each other. Myelination achieves faster conduction by using the myelin sheath to increase the spatial separation of the excitable patches—the nodes of Ranvier. Figure 51-1B shows that, for the same fiber diameter, covering parts of the axon with a myelin sheath forces the excitation current to effectively jump from one node to the next, with very little current lost in the internode because the myelin sheath has a very high membrane resistance and a small electrical capacity.

The geometry of the myelinated fiber is optimized for conduction. Thus, both the myelin thickness and the internodal length bear a relationship to the fiber diameter in such a way as to maximize the conduction velocity at a given fiber diameter (Rushton, 1951; Waxman, 1980). This type of dimensional analysis was pioneered by Rushton (1951), and the most insightful point made concerns the myelin thickness. Rushton argued that, for a given external fiber diameter, the myelin sheath can be neither too thick nor too thin. This can be readily seen from Figure 51-1. At a given outside fiber diameter, thickening the sheath to reduce the current loss across the internode does so at the expense of squeezing the core diameter of the axon, which tends to increase the core resistance and hence impedes the flow of axial current. There is thus an optimal myelin thickness, at a given outside diameter, that optimizes conduction. This myelin thickness is deduced to be 0.3 to 0.4 of the fiber diameter (Rushton, 1952; Waxman and Bennett, 1972), a prediction that is in remarkable agreement with experimental observations. An interesting price that myelination pays is that when compared to a nonmyelinated fiber of the same external diameter, the axon core diameter is always smaller because of the space taken up the myelin sheath (Figure 51-1). Thus, while large myelinated fibers always conduct faster than nonmyelinated fibers, in a very small myelinated fiber, the



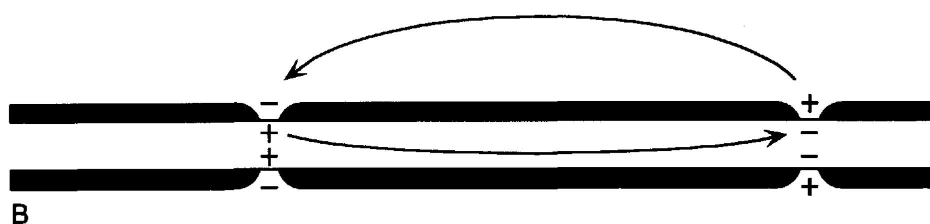


FIG. 51-1. (A) A nonmyelinated fiber and (B) a myelinated fiber illustrating the difference between continuous conduction and saltatory conduction. The two types of fibers are drawn with the same external fiber diameter.

core resistance can become so high that it actually conducts slower than a nonmyelinated fiber of the same fiber diameter. Waxman and Bennett (1972) deduced that it is only above a fiber diameter of 0.2 μm that conduction velocity is increased by myelination. This may explain why in the central nervous system, fibers less than 0.2 μm in fiber are rarely myelinated (Waxman and Bennett, 1972). Interestingly, myelinated fibers less than 1.0 μm in diameter are rare in the peripheral nervous system. This has been attributed by Ritchie (1982), not to a breakdown of Rushton's theory on the relation between myelin thickness and conduction, but rather to the fact that Schwann cells have a minimum size that puts a lower limit on the internodal length in small fibers. When myelinated fibers fall below 1 μm in diameter, the internodal length, which normally should be reduced linearly with diameter to achieve optimal conduction (Rushton, 1951), becomes fixed at the lower limit of the longitudinal length of a Schwann cell. This results in a deviation of the conduction velocity from the optimal value because the internodal length becomes inappropriately too long. Apparently, in the central nervous system in which axons are myelinated by oligodendrocytes, such size limitations on the internodal length do not exist because a single oligodendrocyte myelinates many axons with its fine processes.

Another important consequence of the insulation provided by the myelin sheath is that the metabolic costs of conduction are greatly reduced. The main metabolic cost is incurred not during conduction, which involves ions flowing passively down their ionic gradients, but during the recovery when excess sodium ions gained inside have to be pumped back out against their gradient. The larger the membrane area involved in excitation, the larger the amount of excess ions needs to be pumped out. By restricting excitation along myelinated fibers to tiny nodes of Ranvier, much less pumping of ions is required during the recovery than in nonmyelinated fibers. These two advantages of myelination, speed and efficiency, are best summarized by the following example (Hodgkin, 1964; Hille, 1977). A myelinated frog fiber, with a fiber diameter of 14 μm, has a conduction velocity of ~30 m/s at 24°C. To achieve a similar conduction velocity in a nonmyelinated squid axon, a much larger diameter of 500 μm is required. Conduction in the myelinated fiber involves 5 nodes of Ranvier per centimeter length of fiber, and, based on voltage-clamp data on single myelinated nerves and flux measurements, the myelinated fiber gains 1.3×10^{-16} mol sodium/cm length of fiber for each impulse. In comparison, each impulse in the 500 μm squid axon gains 5×10^{-13} mol sodium/cm. Thus, the metabolic energy needed to pump out the excess sodium ions following excitation is 4000 times larger in a nonmyelinated fiber than a myelinated fiber of comparable conduction velocity.

It is clear that Schwann cells are clearly designed to maximize the conduction velocity of myelinated fibers, as the dimensional analysis clearly indicate. What role is left for Schwann cells to play in saltatory conduction besides insulation? There are many other aspects of myelinated fibers, like the metabolic interactions between Schwann cells and axons, the refractory period, or the ability of the nerve to conduct trains of impulses, which also have important functional consequences on the performance of the system as a whole. Schwann cells do not simply leave the node as a hollow gap in the myelin, but decorate it with a system of intriguing membrane

specialization consisting of gap substance, microvilli, and a dense packaging of mitochondria. William and Landon (1963) first introduced the term "paranodal apparatus" to collectively describe these Schwann cell specialization at the node of Ranvier, and suggest that the paranodal apparatus "could provide a source of energy-rich compounds to fuel ion translocation mechanisms at the nodal axolemma, or play a role in controlling the ionic milieu within the nodal gap (Landon, 1981)." The recent discovery of voltage-sensitive ion channels on the Schwann cell membrane has further strengthened the view that these ensheathing cells can no longer be relegated to a backseat role of passive insulation. Indeed, dynamic ionic interactions between Schwann cells and axons are clearly possible.

Having reviewed the classical, insulation role of Schwann cells, we now turn to the main focus of this chapter, which is on potential new roles of Schwann cells in modulating nerve excitability through the use of voltage-sensitive ion channels expressed on the Schwann cell membrane. We shall only be concerned with Schwann cell-axon interaction in the fast time scale of saltatory conduction. Thus, other intriguing roles for Schwann cell ion channels that have long-term effects on myelinated fibers will not be examined. These include regulation of Schwann cell proliferation by potassium channels during myelin formation and nerve injuries (Konishi, 1989; Wilson and Chiu, 1989; Chiu, 1991), developmental regulation of Schwann cell cytoplasmic volume by potassium channels (Konishi, 1991), and the possible transfer of sodium channels to axons to support channel turnover (Gray and Ritchie, 1985; see Chapter 12, this volume).

How might Schwann cell ion channels affect excitability of myelinated nerves? We believe the key lies in whether ion channels on a Schwann cell are segregated to regions immediately adjacent to the axonal membrane. We will first survey the evidence for channel segregation on Schwann cells. Then we will examine how two types of Schwann cell channels, namely, voltage-sensitive sodium and potassium channels, might be functionally important in modulating nerve excitability.

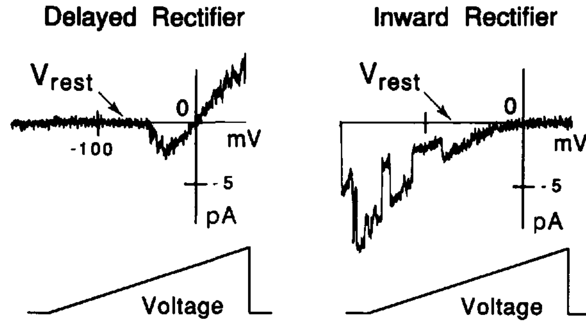
ARE ION CHANNELS SEGREGATED ON MYELINATING SCHWANN CELLS?

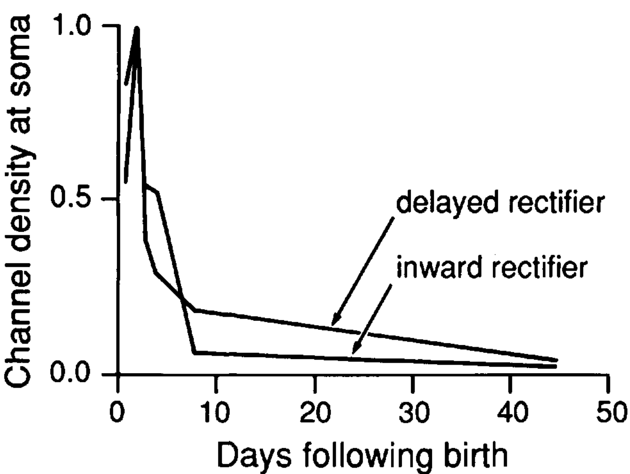
When a Schwann cell elaborates myelin, it greatly increases its own membrane area, and spirals its membrane many times around an axon. Are ion channels expressed uniformly all over the Schwann cell membrane, or segregated to certain regions? An optimal interaction with axons might require relocating channels on Schwann cells to the axon's proximity. The Schwann cell membranes in immediate apposition to the axon consist of the innermost turn of the Schwann cell, as well as the paranodal membranes at a node of Ranvier. Results from patch-clamp analysis of ion channels have pointed towards channel segregation.

Potassium Channels

First hints that potassium channels might be segregated came from patch-clamp studies in which the channel density at the cell body was compared between nonmyelinating and myelinating Schwann cells of adult mammals, and in the myelinating type during early stages of myelin formation. Results from two laboratories have yielded similar results: potassium channels disappear from the cell body during the process of myelin elaboration (Chiu, 1987, Konishi, 1990a, 1990b; Wilson and Chiu, 1990a). Figure 51-2 (top) shows the two types of voltage-sensitive potassium channels recorded from the cell body of Schwann cells at early stages of myelin formation. One is a delayed rectifier, which activates around −40 mV (Konishi, 1990b; Wilson and Chiu, 1990a). The other is an inward rectifier, which is activated by membrane hyperpolarization and passes inward currents better than outward. As myelin is formed, both channels disappear from the cell body (Figure 51-2, bottom two panels).

One explanation for the disappearance of potassium channels from the cell body is that they are relocated to distal myelin processes or inner membrane turns of the Schwann cell (Figure 51-3). Two experimental approaches have been used to test this theory. Konishi (1991) reasoned that these channels should be revealed in whole-cell patch-clamp if the myelin was loosened. By cleverly inducing volume changes, using the water influx that accompanied the activation of the inward rectifiers, Konishi (1991) did find an increase in the size of potassium currents, which was interpreted as arising from channels located in the adaxonal Schwann cell membrane. Wilson and Chiu (1990b) took a more direct approach by placing a patch pipette at the paranodal region. The paranode was first widened with enzymes to make room for the pipette, and upon detecting channels with the pipette, Wilson and Chiu (1990b) proved, through the use of lucifer yellow staining, that the channels originated from Schwann cell membranes rather than from the adjacent, ion channel-enriched nodal membranes. Thus, at a time when potassium channels have all but disappeared from the cell body in adult myelinating cells, these





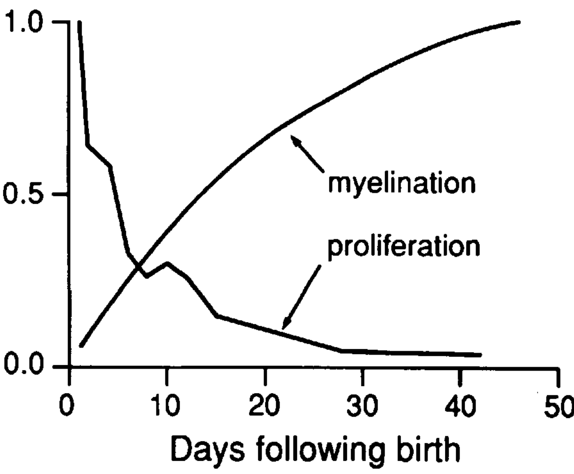


FIG. 51-2. Disappearance of potassium channels from the soma of Schwann cells during myelin formation. **Top:** Single-channel recordings of delayed rectifying potassium channels and inwardly rectifying potassium channels from the soma of neonatal myelinating Schwann cells freshly isolated from rat sciatic nerves. **Middle:** A plot of the relative change in somal channel density for the two potassium channels in myelinating Schwann cells as a function of time after birth. **Bottom:** A plot of the corresponding relative changes in myelination and proliferation. [Modified from Chiu (1991), with permission.]

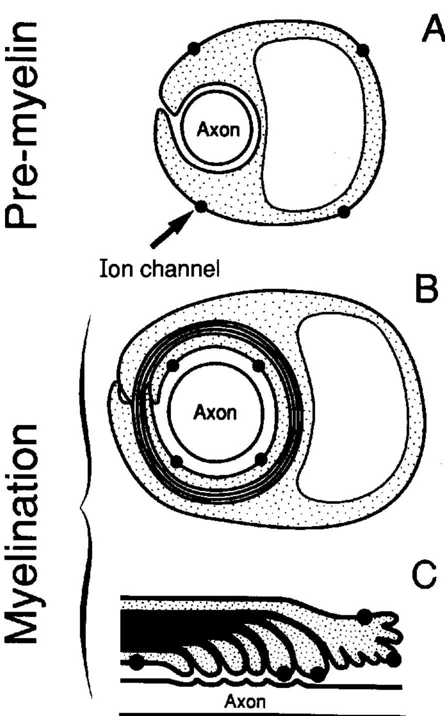


FIG. 51-3. A model for channel segregation in myelinating Schwann cells. (Modified from Chiu (1991) with permission). (**A**) Channels are uniformly distributed in the premyelin state, (**B**) but become relocated to the inner membrane turns, (**C**) or the paranodal processes during myelin formation.

channels (both the delayed rectifiers and inward rectifiers) can be recorded at the paranode. Potassium channels therefore appear to be segregated in mature myelinated Schwann cells. It is important to note that other potassium channel types may be present, and channel segregation of this sort may not apply to all channels. Further, the mechanisms for achieving channel segregation are unclear; they could include dynamic relocation of channels from the soma to the processes (as suggested by the model in Figure 51-3), or a selective downregulation of the number of functional channels at the soma.

Recently, mRNAs for certain types of shaker potassium genes, which encode potassium channels of the delayed rectifying type, have been found to be expressed in rat sciatic nerves (Chiu et al., 1994). Since Schwann cells (presumably the myelinating type) are the major cells in sciatic nerves, the mRNAs are likely to come from myelinating cells. Interestingly, during the first 2 weeks of postnatal development, mRNA levels go up when somal channels are disappearing (Chiu et al., 1994) suggesting channels are synthesized but segregated to extrasomal regions. Recently, antibodies generated against a certain type of delayed rectifying potassium channel (Shaker Kv1.5) were used to directly con-

firm the paranodal localization of potassium channels in Schwann cells of rat myelinated nerves in immunohistochemical studies (Mi et al., 1995).

Sodium Channels

Saxitoxin binding studies have suggested the presence of sodium channels in Schwann cells of rabbit sciatic nerves (Ritchie and Rang, 1983). Patch-clamp studies on freshly isolated Schwann cells from rabbits have revealed voltage-sensitive sodium channels on the cell body of nonmyelinating cells, but not in the myelinating cells (Chiu, 1987, 1993). Hence, like potassium channels, sodium channels also might be segregated to myelin processes during myelin formation. However, immunohistochemical studies with a polyclonal antibody against sodium channels (7437 antisera) shows that antibody staining is present in the cell body cytoplasm, in the cell body plasma membrane, and in the paranodal Schwann cell processes (Ritchie et al., 1990). In another study using an antibody against a conserved region of all known sodium channel genes, Ferguson et al. (1992) have observed that in mouse sciatic nerves, patchy staining is present only in the paranodal regions of Schwann cells. Paranodal patch-clamp studies failed to reveal sodium channels (Wilson and Chiu, 1990b) but this failure might be related to a small sample size. Taken collectively, these various studies suggest that sodium channels are expressed in paranodal regions of Schwann cells, but whether these channels are segregated during myelin formation is still unresolved.

POTASSIUM CHANNELS

Schwann cell potassium channels can modulate excitability of myelinated fibers by regulating the potassium ion concentration in the periaxonal space between the myelin sheath and the axon. Interestingly, a need for potassium homeostasis arises because a mature myelin sheath, though conferring enough insulation for saltatory conduction, nevertheless is leaky to the extent of allowing some degree of activation of axonal channels under the sheath (Chiu, 1991).

How Tight is the Myelin Insulation?

Even though in our earlier review of saltatory conduction the myelin sheath is depicted as a perfect insulator (Figure 51-1), tracer studies have revealed that the periaxonal space between the myelin sheath and the internodal axon can communicate with the extracellular space, either via the Schmidt-Lanterman incisures or the paranodal junction (Hall and Williams, 1971; Mackenzie et al., 1984). Recent electrophysiological studies with single electrodes have demonstrated that during the passage of an action potential, a part of the action currents that flows into the internode takes a short cut in completing the circuit by flowing along the periaxonal space instead of transversing the thick myelin sheath (Barrett and Barrett, 1982). This is not enough to short out saltatory conduction, but clearly allows activation of axonal channels in the internode (see below). Indeed, even the transverse membrane resistance of the myelin sheath is finite to a degree to draw enough current to depolarize the node (Chiu and Ritchie, 1984). There is now mounting evidence that the internodal axolemma produces the resting potential crucial for stabilizing the myelinated nerves (Barrett and Barrett, 1982; Chiu and Ritchie, 1984).

Axonal Ion Channels Under the Myelin Sheath

The portion of a mammalian axon covered by myelin is populated with a rich repertoire of voltage-sensitive ion channels (Black et al., 1990), allowing a dynamic trafficking of ions in the periaxonal space. Fast activating potassium channels are expressed at a high density at the paranodal region and at a low density in both the nodal and internodal axolemma (Brismar, 1980; Chiu and Ritchie, 1980; Grissmer, 1986; Roper and Schwarz, 1989). Slow potassium channels are concentrated at the nodal axolemma and are expressed at a lower density elsewhere (Roper and Schwarz, 1989). Sodium channels have the highest density at the node, and in the internodal region the density drops to about 1/30 of the nodal density (Ritchie and Rogart, 1977; Chiu and Schwarz, 1987; Shrager, 1987).

Evidence that internodal and paranodal ion channels are activated during nerve activity is as follows. Bath application of potassium channel blockers has been shown to induce repetitive activities in myelinated nerves, an observation that has been interpreted as resulting from a removal of the stabilizing effects of paranodal potassium channels (Bowe et al., 1985). Recently, more direct evidence has come from studies in which a single internode is first impaled with a fine electrode to detect a resting potential, then the electrode tip is retracted slightly so that a resting potential is no longer detected. It is argued that the tip has backed into a space between the myelin sheath and the internodal axolemma, namely, a periinternodal space just outside the internodal axon

but completely under the myelin (David et al., 1992). This recording configuration allows a direct monitoring of the conductance of the internodal axolemma during normal nerve excitation, and has provided the strongest piece of evidence that potassium channels in the myelin covered portion of the axon are activated during saltatory nerve conduction.

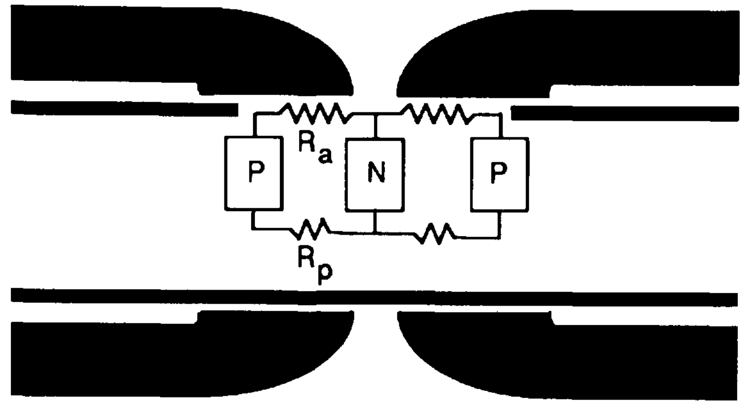
Activation of Axonal Channels Under the Myelin Sheath Can Lead to Significant Potassium Accumulation

The stage is now set for a reasonable estimate of the magnitude of potassium accumulation in the periaxonal space of myelinated nerves (Chiu, 1991). Accumulation is likely to be nonuniform, since both the axonal channel density and width of the periaxonal space are heterogeneous. The periaxonal space is 20 nm along the entire internode proper and narrows to 3 nm along the myelin attachment junction at the paranode. The nodal gap is packed with a system of microvilli that occupies ~80 to 90% of the extracellular space (Berthold and Rydmark, 1983a). Ionic flux is very high at the node due to a high channel density, but a minute internodal flux could also cause significant accumulation because of the smallness of the periaxonal space, particularly at the paranodal junction (see Chapters 26 and 47, this volume). Variations in the series resistance of the periaxonal space can influence the degree of channel activation, hence the degree of potassium accumulation. Taking most of these factors into account, and using voltage-clamp data on channel densities, a computer simulation of potassium accumulation at the node-paranodal region for a large myelinated axon is made (Figure 51-4). The nodal gap is assumed to be 1 μm long and the paranodal segment is 4 μm long. Figure 51-4 shows axonal sodium and potassium currents in the nodal (Figure 51-4A) and the paranodal (Figure 51-4B) segment for a single nonpropagating action potential. Use-dependent accumulation of potassium is shown for the nodal gap (Figure 51-4C) and for the paranodal periaxonal space (Figure 51-4D), with the assumption that potassium clearance occurs only by diffusion. Even though the periaxonal series resistance prevents the paranodal channels from activating to the same extent as the nodal channels, the smallness of the periaxonal space in the paranodal junction, coupled with a relatively long time for diffusion clearance and a high density of delayed rectifying potassium channels at the paranodal axon, results in a use-dependent potassium accumulation that is much higher in the myelin attachment segment than in the nodal gap.

Even though there are theoretical reasons to believe that there is a high accumulation of potassium in the restricted space between the myelin sheath and the axon, this sort of potassium accumulation in myelinated nerves is difficult to verify experimentally. Conventional observations about potassium accumulation in the peripheral nerves, made by placing an ion-sensitive electrode in the extracellular space of rat sciatic nerves, have failed to indicate any significant increases in the extracellular potassium concentration during and following activity (Hoppe et al., 1991). For example, in adult rat sciatic nerves, stimulation could induce increases in extracellular potassium concentration of only several mM at 22°C, and at 37°C this increase rarely exceeds 1 mM (Hoppe et al., 1991). This is in contrast to nerves in the central nervous system where activity induces a much larger elevation in extracellular potassium (Connors et al., 1982). However, as discussed by Hoppe et al. (1991), conventional measurements of extracellular potassium accumulation with ion-sensitive electrodes may underestimate the true accumulation in specialized restricted spaces of myelinated nerves, like those underneath the myelin sheath or at the paranodal junction. Indeed, optical studies on myelinated nerves from the central nervous system, using voltage-sensitive dyes, have suggested that axonal discharge causes a differential accumulation of potassium in a highly restricted space at the paranode (Lev-Ram and Grinvald, 1986).

Do Schwann Cells Play a Role in Potassium Clearance?

The theoretical calculations of Figure 51-4 show that if diffusion is the only clearance mechanism, the extracellular potassium concentration might reach ~10 mM in the narrow ring of paranodal membrane flanking the node following high-frequency nerve transmission. This paranodal accumulation, if not dealt with, will depolarize the node and compromise conduction. In view of the classic role of glia in potassium buffering (Orkand et al., 1966), it is natural to examine how Schwann cells, particularly through the use of potassium channels, may regulate the ionic environment at the paranodal junction. A reasonable scheme is as follows. The inwardly rectifying potassium channels at the paranodal membranes of Schwann cells, by virtue of being maximally activated at the resting potential, might siphon the potassium at the paranodal junction into Schwann cells (Konishi, 1990b; Wilson and Chiu, 1990b). Activation of delayed rectifying potassium channels at a distal site may provide an exit for the incoming po-



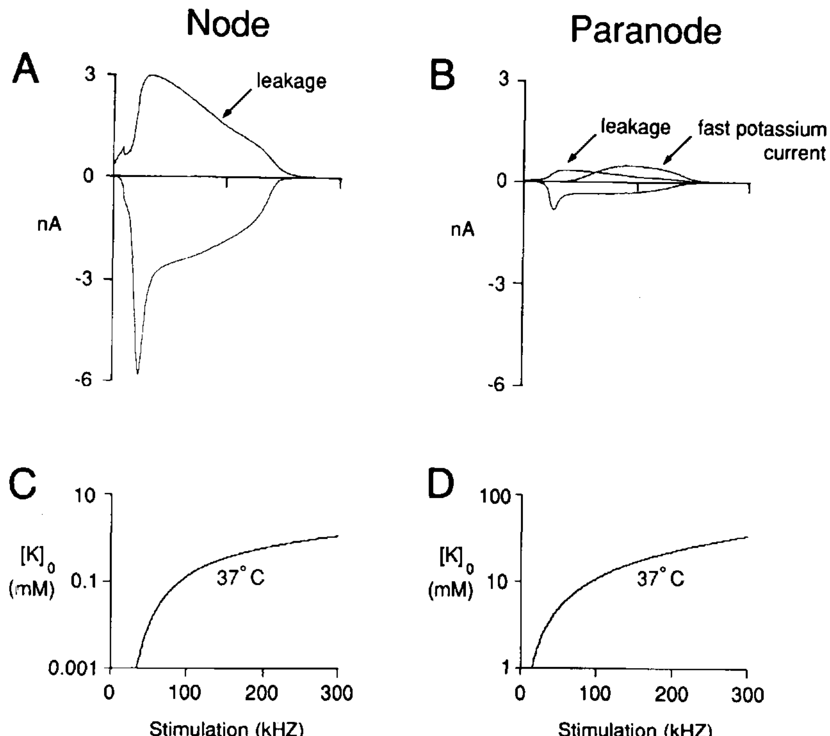


FIG. 51-4. Computer simulations of potassium accumulation in the node-paranode. Top-equivalent circuit used in the simulation. (**A, B**) Ionic currents during a single nonpropagating action potential in the nodal region (*A*) and in the paranodal region (*B*). (**C, D**) Use-dependent accumulation of potassium in the nodal gap (C) and in the periaxonal space between the paranodal axon and paranodal myelin (*D*). Details of computation can be found in Chiu (1991). [From Chiu (1991), with permission.]

tassium ions, allowing for some sort of spatial buffering. The paranodal membrane of Schwann cells apparently is well designed to act as a potassium sink; the Schwann cell:axolemma membrane area at the node-paranodal region is about 10:1, as compared to 1:1 elsewhere along the fiber (Berthold and Rydmark, 1983b).

There is indeed some circumstantial evidence for this scheme. Wurtz and Ellisman (1986) have observed myelin vacuolization and myelin splitting at the paranode during high-frequency stimulation, which might indicate potassium fluxes across the Schwann cell paranodal pockets (Figure 51-6B). A specialization of glial and axon particles at the paranodal junction has been observed, and some of the particles might correspond to potassium channels in-

volved in ionic exchanges (Clayton and Ellisman, 1980). Konishi (1991) observed a swelling of the paranode when Schwann cell inward rectifiers were activated under voltage-clamp, presumably reflecting potassium and water influx. Interestingly, this swelling persists in the absence of external chloride, suggesting that there is little or no chloride movement into Schwann cells for potassium to accumulate at the site of entry. This is an important observation, since it suggests that once potassium ions are siphoned into the Schwann cell at the paranode, they are shunted elsewhere (e.g., exit via delayed rectifiers) rather than accumulated locally. The role of Schwann cells in potassium buffering may be particularly important in neonatal nerves when the myelin sheath is thin and loose and hence activity-dependent potassium accumulation is likely to be more pronounced (Connors et al., 1982). Konishi (1990b, 1991) has suggested that inward rectifiers may be localized to the innermost turn (adaxonal) of the Schwann cell to siphon potassium accumulated along the entire periaxonal space of an immature internode.

One experimental approach to test these proposed roles of Schwann cell potassium channels in potassium buffering involves applying agents to block potassium channels. In the studies of Baker et al. (1987) on rat spinal root myelinated nerves, bath application of 3 to 5 mM Cs^+, a blocker of inward rectifiers, did not impair the firing frequency of the nerves during tetanus, but did lead to a change in the threshold for excitation. In the studies of Hoppe et al. (1991) on rat sciatic nerves, bath application of Ba^{2+}, another potassium channel blocker, did not significantly change the amplitude of stimulated increases in extracellular potassium or the rate of potassium removal. In contrast, Ba^{2+} application led to an increase in activity-dependent potassium accumulation in the central nervous system, suggesting a role for uptake through potassium channels in potassium clearance (Ballanyi et al., 1987). All in all, testing the role of Schwann cell channels in potassium clearance by bath application of potassium channel blockers has two problems. First, since bath application of channel blockers affects both axonal and glial channels, it is unclear how to attribute changes in nerve excitability to a block of glial channels alone. Second, restricted diffusion may prevent bath applied agents to reach and block glial channels expressed in various restricted spaces of a myelinated fiber like the region underneath the myelin sheath. Until a better means becomes available of completely inhibiting Schwann cell potassium channels and does so selectively by not interfering with axonal channels, it would be difficult to assess experimentally the role of Schwann cell channels in modifying nerve excitability. In this respect, a future approach of selectively altering the expression of potassium channels in Schwann cells by transgenic technologies may be particularly useful in elucidating some of the proposed roles for Schwann cells discussed in this chapter.

SODIUM CHANNELS

It has been suggested that sodium channels at Schwann cells or glial processes can create a local depolarization (Gautron et al., 1992) or even trigger a local excitation (Black et al., 1989) that activates a cascade of events important for nodal function. As will be discussed below, the paranodal membranes of Schwann cell, because they are differentiated into various fine compartments with extremely high input resistance, can allow the opening of even a single sodium channel to produce a sizable depolarization. Furthermore, minute Schwann cell pockets or microvilli containing very small volumes of cytoplasm are present at the paranode, and these membrane compartments can function as transduction units to translate brief sodium channel activities into large fluctuations in the concentration of sodium, an important signaling molecule. We will consider these roles of Schwann cell sodium channels by computing the input resistance and sodium loading in various compartments of the paranode.

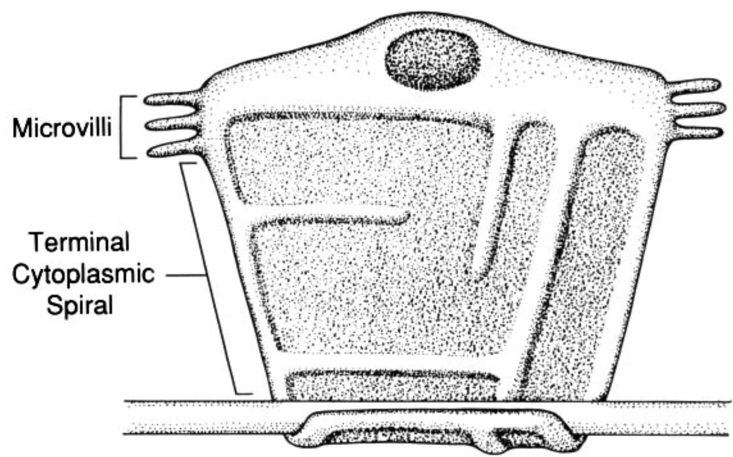
What is the Sodium Channel Density in Schwann Cells In Vivo?

We first need a reasonable value for the sodium channel density for Schwann cells *in vivo*. In freshly isolated Schwann cells from rabbit sciatic nerves, single-channel recordings from the cell body of nonmyelinating Schwann cells have revealed an average of about 2 to 3 overlapping channel openings per excised outside-out patch of area ~2 to 3 μm^2 (Chiu, 1993). This gives a lower limit of ~1 Na channel/μm^2 *in vivo,* at least at the cell body of nonmyelinating Schwann cells. For the case of the myelinating Schwann cells, functional Na channels cannot be detected at the cell body (Chiu, 1987, 1993), but the paranodal regions are strongly stained with antibodies against sodium channels (Ritchie et al., 1990; Ferguson et al., 1992). In the following calculations, we will assume a low sodium channel density of 1/μm^2 for the Schwann membranes at the paranode.

Single Sodium Channel Openings Can Lead to Significant Changes in Membrane Potential and Sodium Concentration in Various Paranodal Compartments of Schwann Cells

In a fully differentiated myelinating Schwann cell, the two paranodal compartments of particular interest are the terminal cytoplasmic spiral and the microvilli (Figures 51-5 and 51-6). Calculations are made of membrane depolarization and sodium loading in these regions by assuming a low sodium channel density of $1/\mu m^2$. To calculate the current generated by a single sodium channel, we assume a single-channel conductance of 20 pS (Shrager et al., 1985; Howe and Ritchie, 1990; Chiu, 1993) and an inward driving force of 100 mV when the channel is activated near the resting potential. Thus, each channel generates 2 pA a piece. Calculations are done for a large myelinated fiber with a fiber diameter of 17.5 μm and associated morphometric data taken from a review chapter by Thomas et al. (Table 1, 1993; the original data was that of Berthold and Rydmark, 1983a).

Terminal Cytoplasmic Spiral. Upon forming compact myelin, the Schwann cell cytoplasm is squeezed and redirected to form thin cytoplasmic tunnels. The terminal cytoplasmic spiral refers to a spiral of cytoplasm at the myelin attachment zone near the node of Ranvier, and is best illustrated by unrolling the myelin sheath from the axon (Fig. 5, top). The terminal cytoplasmic spiral is ~2530 μm long with a circular cross-sectional diameter of ~0.1 μm. When rolled back onto a single axon, this spiral makes 140 turns and comes into close contact over a ~4 μm length of paranodal myelin attachment zone. The cytoplasmic pockets seen hanging over the paranodal myelin attachment zone in fact come from the same cytoplasmic spiral (Figure 51-6B). Because this terminal cytoplasmic spiral is formed by myelin compaction, it is more or less electrically isolated from the rest of the cell, and thus can be treated as an



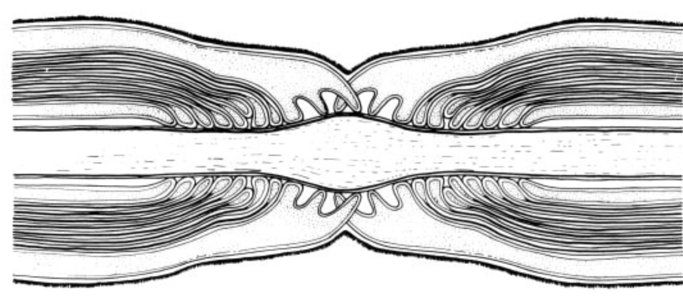
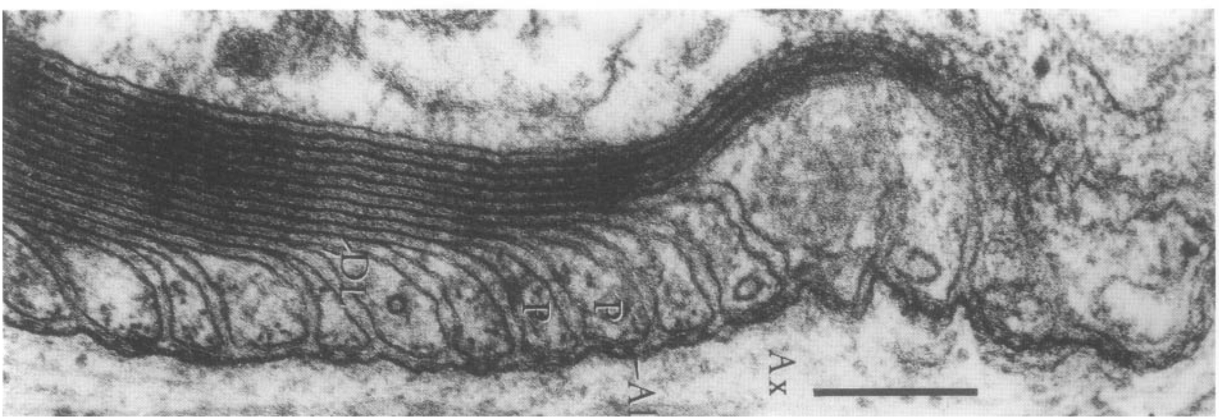


FIG. 51-5. A schematic drawing of the unrolling of a mature myelin sheath from an axon. **Top:** the unrolled sheath, showing the transverse and the longitudinal Schmidt-Lanterman incisures. Note the microvilli and the terminal cytoplasmic spiral. **Bottom:** when rolled back around the axon, the terminal cytoplasmic spiral runs around the paranodal attachment segment many times, creating Schwann cell pockets overhanging the paranodal axon.



B

FIG. 51-6. Electronmicrographs of (A) Schwann cell microvilli and (B) pockets of the terminal cytoplasmic spiral at the paranodal region. *A:* cross-sectional view at the node of Ranvier from a cat nerve. Bar = 0.5 μm. [From Berthold and Rydmark (1983a), with permission.] *B:* longitudinal view at the paranodal region. Sciatic nerve of an adult rat. Bar = 0.2 μm. [From Fig. 6-15 of Peters et al. (1991), with permission.]

infinite cable 0.1 μm in diameter using appropriate cable equations (Jack et al., 1975). Hille (1970) has pointed out that in small axons, the input resistance is so large that opening of a single sodium channel can lead to a large depolarization.

Table 51-1 shows a calculation of the input resistance of the terminal cytoplasmic spiral by assuming two values for the resting membrane resistivity for Schwann cells. One value (24,700 Ωcm^2) is calculated from the whole-cell clamp data of Howe and Ritchie (1990) on cultured mammalian Schwann cells, while the other value (1000 Ωcm^2) is typical for most biological membranes, and has been suggested to apply also to squid Schwann cells (Villegas, 1972). The calculated input resistance of the terminal cytoplasmic spiral, R_{input}, is extremely large, being 1.7×10^{10} Ω or 3.4×10^9 Ω for a 0.1 μm diameter spiral. As the spiral diameter is shrunk to 0.05 μm (see Figure 51-6B), the input resistance increases by 2 to 3 fold.

Table 51-1 shows the calculated local depolarization produced by one sodium channel that opens for a brief 1 ms. For the 0.1 μm spiral it is 6 to 7 mV per channel per millisecond, and for the 0.05 μm spiral it is 18 to 21 mV per channel per millisecond. Since inward rectifiers in Schwann cells are downregulated following axonal deprivation (Konishi, 1992), we believe that the membrane resistivity of 24,700 $\Omega\ cm^2$, which is taken from cultured cells, may be too high for *in vivo* Schwann cells. Hence in the following discussion, we will use parameters based on a membrane resistivity of 1,000 Ωcm^2.

Could depolarizations from individual sodium channels sum when several channels are opening simultaneously? This depends on the spacing of sodium channels along the spiral relative to the space constant of the terminal cytoplasmic spiral. Table 51-1 shows the calculated spacing between sodium channels, assuming a low density of $1/\mu m^2$, as well as the space constant of the terminal cytoplasmic spiral. The values in Table 51-1 allow a calculation of the size of the depolarization when multiple channels are open. A reasonable scenario is the activation of one turn's worth of sodium channels around the paranodal axon. For the terminal cytoplasmic spiral with an input resistance of 3.4×10^9 Ω, a space constant of 32 μm, and a spacing of 3.2 μm between sodium channels, one turn of the spiral around the paranodal axon (circumference ~15 μm) will have ~5 Na channels. If these five Na channels are to open simultaneously for 1 ms, each creating a local depolarization of 6 mV, the peak depolarization can be calculated by superimposition, and will equal $6 + 2*6*\exp[-3.2/32] + 2*6*\exp[-2*3.2/32] = 25$ mV, if a change in driving force upon depolarization is ignored.

The fact that a few sodium channels can depolarize the spiral by ~25 mV, the threshold of firing in most axons, raises the possibility of regenerative responses in the paranodal spiral. It should be noted that small neonatal optic nerve axons with a diameter of 0.2 μm can conduct action potentials with a sodium channel density as low as $2/\mu m^2$ (Waxman et al., 1989), and it has been suggested that a high input resistance is one reason for making conduction possible by allowing a few sodium channels to produce a large depolarization. Even before the demonstration of sodium channels in the paranodal regions of Schwann cells (Ritchie et al., 1990), Ellisman et al. (1980), based on the observed richness of sodium and calcium ions in the paranodal Schwann cell pockets, have already postulated the possibility that the terminal cytoplasmic spiral might be involved in excitation. The use-dependent changes in the structures of paranodal myelin loops may reflect some sort of propagating responses in the terminal spiral (Wurtz and Ellisman, 1986).

Now consider changes in sodium concentration in the terminal spiral. Activating one turn of the cytoplasmic spiral (flicking open 5 channels for 1 ms, each generating ~2 pA a piece) would generate a net influx of $\sim 6 \times 10^4$ Na ions. The volume of one turn of the spiral, 15 μm long and 0.1 μm in diameter, is $\sim 0.1\ \mu m^3$. The intracellular sodium concentration thus increases by ~1 mM. Further, the volume of the immediate extracellular space surrounding one turn of the spiral, with a 200 A space between the pockets, is approximately equal to the intracellular volume. This means that the extracellular sodium concentration should also drop by an equal amount. In total, activating only 5 sodium channels along one turn of the spiral (15 μm long) can alter the transmembrane sodium gradient by ~2 mM or 1 to 2% of the normal gradient. Thus, repetitive paranodal activities could easily lead to significant changes in spiral sodium. A dynamic redistribution of cation binding sites (thought to be reservoirs for sodium ions) between the node and the paranode has been observed during electrical stimulation (Zagoren and Arezzo, 1982).

Paranodal Schwann Cell Microvilli. There are about 800 Schwann cell microvilli in the nodal gap of a large fiber. Each microvillus is 0.084 μm in diameter and 1 μm long. A cross-sectional view at the node (Figure 51-6A) shows that the microvilli radiate toward the axolemma surface. The total membrane resistance of one villus is very high, probably on the

TABLE 51–1. *Input Resistance of the Terminal Cytoplasmic Spiral*

D (μm)	R_m (Ωcm^2)	l (μm)	t_m (ms)	R_{input} (Ω)	Depolarization by one Na channel opening for 1 ms (mV)	Spacing Na channel (μm)
0.10	24,700	158	25.0	1.7×10^{10}	7.0	3.2
	1,000	32	1.0	3.4×10^{9}	6.0	3.2
0.05	24,700	122	25.0	5.2×10^{10}	21.0	6.4
	1,000	25	1.0	1.0×10^{10}	18.0	6.4

D: diameter of the terminal cytoplasmic spiral (see Figure 51-5). The value of 0.1 μm is taken from Thomas et al. (1993, Table 1). The value of 0.1 μm is the normal value. Calculations also have been done for $D = 0.05$ μm to show the effects of a smaller spiral on the different parameters.

R_m: The assumed specific membrane resistance (Ωcm^2) for the Schwann cell membrane. The value of 24,700 Ωcm^2 is calculated from whole-cell patch clamp data on cultured mammalian Schwann cells (Howe and Ritchie, 1990). From table 1 of Howe and Ritchie (1990), the input resistance is 1.54 GΩ and somal capacity is 16.5 pF for myelinating Schwann cells cultured from adult rabbit sciatic nerves. Assuming a value of 1 μF/cm^2, this gives 24,700 Ωcm^2. The value of 1000 Ωcm^2 is taken from data on Schwann cells of the squid giant axon (Villegas, 1972).

l: Space constant of the terminal cytoplasmic spiral. The cytoplasmic spiral is approximated to be an infinite cable (Jack et al., 1975).

$$l = \left(\frac{r_m}{(r_a + r_0)}\right)^{1/2}$$

$$r_m = \frac{R_m}{\pi D},$$

$$r_a = \frac{R_a}{\pi (D/2)^2},$$

$$r_0 = \frac{R_0}{\pi D W},$$

where r_m is the membrane resistance per unit length, r_a is the intracellular resistance per unit length, and r_0 is the extracellular resistance per unit length. The specific membrane resistivity, R_m, is either 24,700 Ωcm^2 or 1000 Ωcm^2. The specific resistivity for the intracellular pathway, R_a, is assumed to be 166 Ωcm (Neumcke and Stampfli, 1982). This value is from rat sciatic nerves, and is higher than the typical frog value of 110 Ωcm; the mammalian value is considered more appropriate here. The specific resistivity for the extracellular pathway, R_0, is assumed to be 65 Ωcm (Barrett and Barrett, 1982). The extracellular resistance is calculated only for the immediate extracellular space surrounding one cytoplasmic pocket (see Figure 51–6B). This space, W, is taken to be 200 A. This value is obtained by surveying published micrographs from various laboratories [~240 A from Fig. 2 of Ellisman et al. (1987); ~260 A from Fig. 3–17 of Thomas and Ochoa (1984), ~200 A from Fig. 2 of Raine (1982); ~180 A from Fig. 6–15 of Peters et al. (1991)]. Note that immediately facing the axon, the space between the cytoplasmic spiral and the axon can narrow to ~3 to 5 nm.

t_m: membrane time constant, being equal to $R_m \times C_m$, where $C_m = 1$ μF/cm^2.

R_{input}: The calculated input resistance of the terminal cytoplasmic spiral. Note the input resistance is calculated for the case of the spiral wrapped around the paranodal region, and the calculation has taken into account the resistance in the extracellular space between adjacent turns.

$$R_{input} = \frac{(r_m\, r_a)^{1/2}}{2}$$

Depolarization per Na channel per millisecond: This column gives the local depolarization produced by one sodium channel opening for 1 ms generating 2 pA of inward current near the resting potential (see text). The voltage response is that of an infinite cable in response to a point application of a current step 2 pA in amplitude and 1 ms in duration. We estimate this voltage response from Fig. 3.3 of Jack et al. (1975) which gives the time course of the voltage change in an infinite cable subjected to a step current of infinite duration. The steady-state voltage is 2 pA $\times$ R_{input}. For case where the membrane time constant is 1 ms, the voltage at 1 ms is exactly 84% of the steady value. For the case where the membrane time constant is 25 ms, the voltage at 1 ms is ~20% of the steady value.

Spacing between Na channels: this spacing along the cytoplasmic spiral is calculated based on a density of 1 channel/μm^2.

order of ~10^{11} Ω. The intracellular resistance along the length of one microvillus is ~10^8 Ω, and connects the cytoplasm of each villus to the rest of the Schwann cell proper whose uncompacted membrane area is several orders of magnitude larger than the villus area. This suggests that the effective input resistance of one villus is approximately equal to 10^{11} Ω and 10^8 Ω in parallel, which is thus namely ~10^8 Ω. A complete equivalent circuit analysis of the microvilli is needed to substantiate this statement. Nevertheless, this suggests that the input resistance of a villus is at least one order of magnitude smaller than that of the cytoplasm spiral (10^9–10^{10} Ω, Table 51-1), suggesting a much smaller depolarization per sodium channel. However, given the intense sodium channel staining of the microvilli (Ritchie et al., 1990), a large depolarization is still possible with a high sodium channel density.

As for sodium loading in the microvilli, with each villus having a volume of ~0.005 μm^3, the opening of one sodium channel for 1 ms will increase intracellular sodium by ~4 mM. This increase is likely to be very transient, since sodium ions will diffuse immediately, along a short distance of ~1 μm, from the villus to the Schwann cell proper which acts like a large sink. Nevertheless, a repetitive activation of microvilli is likely to lead to large transient increases in intracellular sodium.

What Might Activate Sodium Channels in Schwann Cell Paranodes? Any substances released by the axon that can depolarize the Schwann membrane may result in sodium channel activation. A prime candidate is potassium ions released at the node-paranode regions during repetitive activity. Interestingly, computer simulations (Figure 51-4) suggest that activity-dependent potassium accumulation is higher in the paranodal periaxonal space than in the nodal gap, suggesting that the terminal cytoplasmic spiral (Figure 51-6B), rather than the microvilli (Figure 6A), will be differentially activated by potassium. Another candidate is a diffusable neuroactive substance (other than potassium) released by axons. For example, adenosine (Maire et al. 1984) and glutamate (Weinreich and Hammerschlag, 1975) are known to be released from frog and mammalian fiber tracts during repetitive nerve stimulation. Mammalian Schwann cells in culture express various neuroligand receptors (Lyons et al., 1992; Yoder et al., 1992), some of which could mediate membrane depolarization. In squid, axonal stimulation leads to complex changes in the membrane potential of Schwann cells via activation of glutamate and acetylcholine receptors (Evans et al., 1991).

Possible Physiological Roles of Activation of Schwann Cell Sodium Channels. First, sodium channels can be used to amplify voltage. The high input resistance of the terminal spiral permits the generation of large membrane depolarizations with a low sodium channel density. For instance, an initial potassium-mediated depolarization of the terminal spiral can be amplified significantly by recruiting just a handful of sodium channels around one spiral turn. Even larger depolarizations can be achieved by activating more spiral turns (e.g., using Table 51-1, it can be calculated that a 100-mV depolarization can be achieved by activating ~5 to ~35 channels, corresponding to ~2 to ~9 spiral turns, depending on the spiral diameter. The depolarization is an underestimate since a reduction in driving force is ignored when the membrane is depolarized). Any shrinkage of the spiral will increase the input resistance and augment this voltage amplification. For example, a 50% reduction of the spiral diameter from 0.1 μm to 0.05 μm (some pockets in Figure 6B have diameters of ~0.05 μm) will increase the depolarization per sodium channel by 300% (Table 51-1). Voltage amplification based on high input resistance allows the efficient use of a small number of sodium channels to generate large membrane depolarizations to drive or modulate certain membrane processes, like the delayed rectifying potassium channels (whose activation can provide an exit for potassium ions siphoned in through the inward rectifiers) or electrogenic carriers.

Second, use-dependent alterations of sodium gradients across Schwann cell membranes could modulate the net flux of substances through transporters whose operation is coupled to sodium ions. Transporters of amino acids are of particular interest. For example, the transporter for glutamate is coupled to sodium and potassium (Nicholls and Attwell, 1990), is electrogenic, and normally transports glutamate into cells. Under conditions of elevated internal sodium, elevated external potassium, and large membrane depolarizations, glutamate can be released by reversal of the uptake carrier. Other sodium-coupled carriers include those which transport adenosine across cell membranes. Schwann cells might modulate nodal excitability by releasing substances triggered partly by a change in sodium gradient. It should be pointed out that axons do appear to have receptors. The nodal membrane is richly stained with alpha-bungarotoxin (Freedman and Lenz, 1980), and could be the target for acetylcholine released from Schwann cells (Evans et al., 1991). Adenosine receptors have been suggested to be present on axons of myelinated nerves whose activation

may modulate sodium channels via cAMP dependent phosphorylation (Ribeiro and Sebastiao, 1987).

Besides amino acid transporters, two other carriers are worth mentioning. One is the Na/K ATPase, found to be located on the Schwann cell microvilli (Ariyasu et al., 1985). Use-dependent loading of sodium in the microvilli through sodium channels could be used to regulate the activity of this enzyme (Black et al., 1989; Gautron et al., 1992). Activation of the Na/K ATPase may assist potassium buffering by transporting potassium into the microvilli. Another carrier is the Na/Ca exchanger. This exchanger can be driven to deliver a net influx of calcium into the microvilli or the cytoplasmic spiral given the right sodium loading and large depolarizations. Confocal imaging analysis has shown that axonal activation leads to a calcium elevation in the paranodal Schwann cell cytoplasm, but this calcium change is suggested to be linked to a ryanodine receptor (Lev-Ram et al., 1992). It is interesting that Ca-ATPase is located primarily in the paranodal loops, suggesting a requirement for calcium homeostasis in this region (Mata et al., 1988). Intracellular calcium elevation in the paranodal apparatus may trigger calcium-dependent release of substances from Schwann cells, or have metabolic consequences like modulation of the glycogenloytic pathway (Ververken et al., 1982).

SUMMARY

Schwann cells of peripheral axons not only provide the insulation needed for saltatory conduction, but, as we have argued in this chapter, also can actively modulate nerve excitability through the use of voltage-sensitive channels expressed in the immediate vicinity of the axons. Potassium channels expressed at the paranodal region of Schwann cells could have a critical role in saltatory conduction by regulating the potassium concentration in the space between the myelin and the axon. A reasonable buffering scheme is one in which potassium is siphoned in through inward rectifiers, and exits distally through delayed rectifiers. We have also argued that the Schwann cell membranes at the paranode, because of their differentiation into regions of high input resistance and small cytoplasmic volumes, should, in principle, allow robust voltage and sodium signaling to occur with an extremely low density of sodium channels. Thus, a low density of voltage-sensitive channels on Schwann cells need not preclude these channels from exerting profound physiological effects on the cells. Sodium channel mediated signaling could drive various sodium-coupled, electrogenic membrane carriers that might affect nodal excitability through ligand release or modulation of the ionic environment. It is likely that an elucidation of these new roles for Schwann cells in saltatory conduction may require new techniques, like the use of transgenic animals in which the expression of Schwann cell channels can be selectively altered.

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52 Glial cells and the supply of substrates of energy metabolism to neurons

JONATHAN A. COLES

Golgi, in 1886, and many others since then, have drawn attention to the fact that, since astrocytes are interposed between the pericapillary space and the neuronal membranes, and their endfeet envelop the capillaries, they may be part of the route by which metabolic substrates reach the neurons (see Chapter 32). This chapter limits itself to substrates of energy metabolism; in other words, how the glucose that passes out of capillaries ends up as carbon fuel in the neurons. Four possibilities that will be considered are illustrated in Figure 52-1. First, the astrocytes may play no role: the glucose simply diffuses round them (Figure 52-1A). Second, glucose may pass through astrocytes rather than through extracellular space on its way to the neurons (Figure 52-1B). Third, glucose may enter the astrocytes at their endfeet and be transformed into a different substance which is transferred to the neurons (Figure 52-1C). Fourth, glucose may enter astrocytes and be transformed into glycogen, which is broken down to supply substrate to the neurons when demand exceeds supply (Figure 52-1D). These possibilities are not mutually exclusive, and in mammals there is some evidence for the three processes A, C, and D; process B cannot be excluded (processes are indicated in Figure 52-1).

Much useful work on the subject has been done on invertebrates, some of it involving cells not originally called glial cells. The term *glial cells* is therefore used here to mean those cells with membranes close to neurons that appear to play no direct role in electrical signaling but may supply nutrients.

NO ANATOMICAL NEED FOR SUBSTRATES TO PASS THROUGH GLIAL CELLS THAT ENVELOP NEURONS

In the leech ganglion, solutes from the blood can reach the neurons by diffusing through extracellular clefts (Nicholls and Kuffler, 1964) (Figure 52-2A), but in most higher animals a blood-brain barrier is formed by junctions between a layer of cells of another type, through which substrates must pass. These barrier cells have different origins in different classes. In higher vertebrates they are the capillary endothelial cells (see Chapter 50) (Figure 52-2D). In insects, elasmobranchs and cephalopods the cells that form the blood-brain barrier are of glial origin (see Chapter 6) (Figure 52-2B, C); but, although substrates probably must pass through them, these barrier glial cells will not be considered here. We are concerned with glial cells, such as the astrocytes in vertebrates, or outer pigment cells in the bee retina, whose processes reach out to envelop neurons. These cells appear not to constitute a barrier to extracellular diffusion. Brightman and Reese (1969) concluded from electron microscope studies that molecules as large as ferritin can diffuse through the extracellular clefts between the pericapillary endfeet of mammalian astrocytes. In bee retina, where each cluster of sensory neurons is completely surrounded by a cylinder of glial cells, tetrodotoxin can pass rapidly through the glial cylinder to reach the neurons (Coles and Schneider-Picard, 1989a); it is probable that this substance diffuses through the extracellular clefts between glial cells rather than crossing glial cell membranes. Hence, as Kuffler and Nicholls (1966) concluded, there is no apparent *anatomical* necessity for metabolic substrates to be delivered to neurons by the adjacent glial cells.

UPTAKE OF GLUCOSE INTO NEURONS AND GLIAL CELLS

If substrates of energy metabolism are supplied to the neurons by glial cells, then the glial cells must first take up these substrates (or their precursors) in quantities sufficient for their own needs and those of the neurons as well. It is therefore of great interest to compare the uptake of substrates by glial cells and by neurons. In mammals over 95% of the substrate for brain work crosses the blood-brain barrier as glucose, so this is essentially a question of glucose uptake (see Lund-Andersen, 1979). Glucose may not be the main substrate in all species: in cockroach

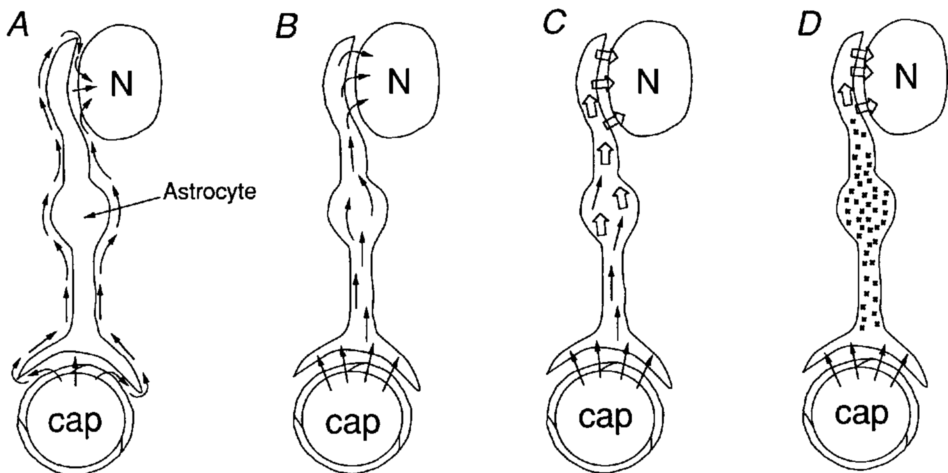


FIG. 52-1. Four hypothetical ways by which glucose leaving a capillary (*cap*) might end up as carbon fuel in a neuron (N). (A) Diffusion through extracellular clefts. (B) Movement of glucose through an astrocyte. (C) Glucose enters an astrocyte and is converted to another substance which is supplied to the neuron. (D) Glucose is used to make glycogen in the astrocyte; glycogen is broken down to provide a substrate for the neuron. *Thin arrows,* glucose; *fat arrows,* another substance, possibly alanine or lactate; *small crosses,* glycogen. Present experimental evidence does not clearly exclude any of the schemes.

(*Periplaneta americana*) where the hemolymph contains a high concentration of trehalose, a dimer of glucose, this appears to be the major substrate, even though glucose crosses the blood-brain barrier more readily (Treherne, 1960). However, most experimental studies on the uptake of carbohydrate have been devoted to glucose.

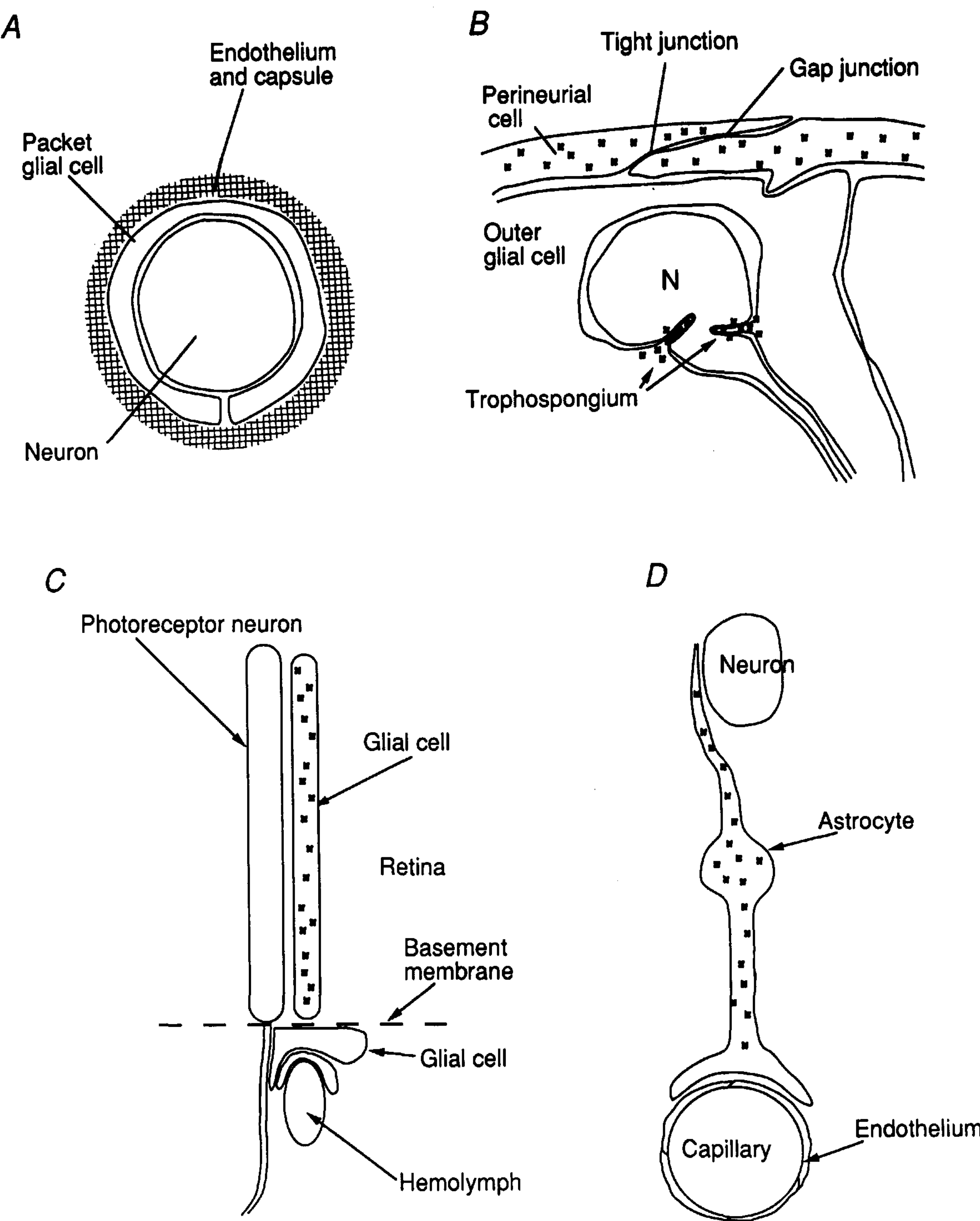
The question of which cells take up glucose that has been supplied by the blood is not easily resolved experimentally. Uptake studies can conveniently be performed on cells in culture, but the results may be misleading. Cells that were unable to take up the essential substrates provided in the culture medium would not survive, and would be automatically excluded from the study. Expression of many genes is known to be stimulated by the serum that is routinely included in culture media (e.g., Barres et al., 1989), and expression of the gene for the rat brain glucose transporter in neuronal cultures is stimulated by such factors as insulin and insulinlike growth factor-1 (Werner et al., 1989). Cell cultures are nearly always derived from fetal or neonatal tissue in which glia-neuron interactions are not fully developed (e.g., Ransom et al., 1985), and the neurons may obtain their metabolites differently from neurons in mature tissue. It is therefore preferable to look at glial cells and neurons either *in situ* or immediately after isolation. The first study of the iden-

FIG. 52-2. Simplified schemes of the routes from blood to neuron in four tissues. *Small crosses* indicate glycogen. (A) Leech neuron. Diagram based on that of Kuffler and Nicholls (1966) to illustrate the presence of diffusion pathways from the blood through clefts in the packet glial cells to the neurons in ganglia of leech ventral nerve cord. The full anatomy of the ganglion is given by Coggeshall and Fawcett (1964), Schlue et al. (1980), and Schlue and Deitmer (1980). Glycogen distribution, which is variable, is *not shown:* see the sections *In Leech Ganglion both Neurons and Glial Cells form Glycogen from Glucose* and *Invertebrate Ganglia.* (B) Insect ventral nerve cord. Having passed the blood-brain barrier formed by a layer of perineurial cells round the ganglion, substances must pass between or through the outer glial cells which surround the neuronal cell bodies (*N*); the inner glial cells are beyond the bottom of the figure. Based on Wigglesworth (1960) and Treherne and Scholfield (1981) for the ventral ganglia of cockroach (*Periplaneta americana*). (C) Bee retina. The retinas of the compound eyes of insects contain only one class of neuron, the photoreceptors. The retina of the drone is particularly large and simple. The elongated cell bodies of the photoreceptors extend for about 500 μm from the dioptric apparatus associated with each corneal facet to the basement membrane that forms the proximal boundary of the retina. Situated between the photoreceptors and lying parallel to them over their length are the glial cells known as "outer pigment cells." The basal membrane has the appearance of collagen. It is penetrated by the axons of the photoreceptors and by tracheoles. No blood vessels penetrate the retina; hemolymph channels run close to the proximal face of the basal membrane and are each surrounded by another class of cell called "pigmented glial cell" by Shaw (1978). The blood-retina barrier is at this level. Figure based on Perrelet (1970), Shaw (1978), and unpublished observations. (D) Vertebrate brain. The blood-brain barrier is situated at the capillary endothelium (Reese and Karnovsky, 1967). Astrocytes, radial glia and tanycytes have endfeet apposed to the capillaries (Chapters 1 and 4, this volume), but substances can diffuse freely through the extracellular clefts between the endfeet.

tity of the cells that took up glucose was made in the leech ganglion.

In Leech Ganglion Both Neurons and Glial Cells Form Glycogen from Glucose

Wolfe and Nicholls (1967) incubated leech ganglia in glucose-6-^{3}H for 10 minutes, washed them in inactive glucose for a further 10 minutes, and found that 17% of the radioactivity was in glycogen. They studied the cellular distribution of the newly formed glycogen by autoradiography. The perikarya of the neurons were densely marked, much more so than the cytoplasm of the surrounding packet glial cells; the neuropil glial cells were heavily marked. A freshly isolated neuron also took up glucose and formed glycogen. These results suggest that both neurons and glial cells took up glucose from the extracellular fluid. Using a technique



that is basically similar, but supplying the glucose analogue 2-deoxy-D-glucose rather than glucose itself, Pentreath and Kai-Kai (1982) found that radioactive glycogen was predominantly, but not exclusively, in the packet glial cells. It is difficult to reconcile these results. The species were similar but not identical (*Hirudo medicinalis* and *Haemopis sanguisuga*), and this could conceivably account for the difference. Alternative possibilities are that somehow the biochemical pathways allow glucose, but not deoxyglucose, to form glycogen in the neurons, or that the animals had been fed differently.

Glucose Uptake in Other Species

Cellular Localization of Glucose Uptake by 2-Deoxyglucose. 2-Deoxy-D-glucose (DG) is transported into cells by glucose transporters and converted mainly to deoxyglucose-6-phosphate (DG6P), with up to 20% appearing in other deoxyglucose phosphates (Dienel and Cruz, 1993). Further reactions are almost completely blocked, although a small amount can be incorporated into glycogen (1 to 10% with typical labeling protocols) (Pentreath et al., 1982). Injection of [^{14}C]DG into the bloodstream is followed by accumulation of [^{14}C]DG phosphates in the cells that took up glucose and the regions of brain that took up most glucose can be found by autoradiography of tissue sections (Sokaloff et al., 1977). The method, as normally used, lacks the spatial resolution necessary for identifying the cells that contain the marker substance. Resolution at a cellular level has been achieved in a few tissues by exposing them to a high concentration of [^{3}H]DG rather than [^{14}C]DG, or by using enzymatic analyses, as now described.

In Insect Retina, Glucose Is Selectively Taken Up by the Glial Cells. In the retina of the drone honeybee, the regularity of the arrangement of the neurons (photoreceptors) and the principal glial cells ("outer pigment cells") allows them to be distinguished in autoradiographs of cross sections. Tsacopoulos et al. (1988) incubated slices of retina in medium containing [^{3}H]DG for 60 minutes, washed for 30 minutes, then froze, lyophilized, and embedded the retina. The labeling was intense in the glial cells and undetectable in the neurons, even though these neurons consume far more energy than the glial cells do and have only limited reserves of carbohydrate (Tsacopoulos et al., 1983) (see the section Drone Retina). Earlier results by Buchner et al. (1984) on *Drosophila* that were fed [^{3}H]DG suggested that the same was true in this species.

In this key experiment, the photoreceptors must have been taking up some substrate of energy metabolism, but they did not take up glucose from the incubation medium. Since no other exogenous substrates were supplied, they were presumably receiving a substrate from the glial cells, and this substrate was not glucose. (Figure 52-1C).

Vertebrate Retina. The experimental accessibility and well-defined cell types made the retina the piece of vertebrate nervous tissue most promising for the identification of the cells that take up glucose. Basinger et al. (1979) incubated pieces of goldfish retina with 2 mM glucose and [^{3}H]DG, fixed them with glutaraldehyde, and embedded them. In dark-adapted retinas most of the photoreceptor neurons were labeled, and the labeling was less in light-stimulated retinas; this correlates with the way membrane currents in vertebrate photoreceptors are reduced by light. Labeling was also found in bipolar cells and horizontal cells (both neurons) of types that were likely to have been depolarized by the light stimulation. Witkovsky and Yang (1982) in isolated frog retina and Sperling et al. (1982) in monkey found densest labeling in the photoreceptors. In all these experiments the DG was supplied from a bath, and not by way of the blood vessels, so it is conceivable that the supply pathways differed from those *in vivo*. This objection was overcome by Morjaria and Voaden (1982), who injected [^{3}H]DG into the bloodstream of rats and, although unable to do autoradiography, separated the photoreceptors from the rest of the retina before measuring the radioactivity. They found that in retinas of dark-adapted rats nearly half the total radioactivity was in the photoreceptors. The photoreceptors of light-adapted rats were less labeled. This result is strongly in favor of direct uptake by neurons, although it cannot be excluded that DG passes into and then out of the retinal astrocytes without being phosphorylated and trapped (Figure 52-1B).

In contrast to these results favoring neuronal uptake, Poitry-Yamate and Tsacopoulos (1991), who dissected retinas of guinea pig under dim white light, and exposed them to [^{3}H]DG in the dark, found, in autoradiographs of frozen-dried and embedded sections, that the labeling was concentrated on the Müller glial cells, with no detectable labeling of photoreceptors or ganglion cells. In subsequent experiments, Poitry-Yamate and Tsacopoulos (1992) demonstrated the uptake of glucose into freshly isolated Müller cells and its conversion to glucose-6-P.

Mammalian Brain. Akabayashi and Kato (1992, 1993) used a variant of the usual DG method. They injected rats with nonradioactive DG, froze them,

dissected out single cells, and measured the cellular content of DG and DG6P by an enzymatic method. Although they did not report values for glial cells *per se,* they found that neuronal cell bodies from the dorsal horn had higher uptakes than did adjacent neuropile, which presumably included glial cell processes.

Conclusions Concerning the Cellular Localization of Glucose Uptake

In bee and fly retinas, glucose is taken up almost exclusively into glial cells. In vertebrate tissues the majority of papers report uptake predominantly into neurons, the glucose marker being supplied via the capillaries in the case of the work by Morjaria and Voaden (1982) and by Akabayashi and Kato (1992, 1993). In contrast, the work of Poitry-Yamate and Tsacopoulos (1991, 1992) favors glial uptake. A consistent pattern in the published work is that, with the exception of Wolfe and Nicholls (1967), all authors appear to have found results that fitted their expectations.

BRAIN GLUCOSE TRANSPORTERS

Knowledge of the cellular localisation and properties of glucose transporters in the brain would provide important information about the delivery of substrates to neurons. There is, general agreement that movement of glucose across the membranes of isolated brain cells, like that across the blood-brain barrier (Crone, 1965), is mainly by facilitated transport, that is, it is not coupled to some source of energy other than its concentration gradient (see Bachelard, 1975). Recently, there has been a rapid increase in knowledge about glucose transporters in the brain. The technique has been to screen brain cDNA libraries to find genes similar to those expressing previously known glucose transporters (for reviews see Silverman, 1991; Baldwin, 1993). The two principal glucose transporters found in this way in rat brain are:

1. The erythroid/brain glucose transporter, designated GLUT1. It is expressed in high levels in brain microvessels (Kasanicki et al., 1987) and has been reported in both neurons and glial cells in culture (Werner et al., 1989; Sadiq et al., 1990).
2. The transporter GLUT3, also sometimes known as the brain glucose transporter, is abundant in adult brain (Nagamatsu et al., 1992). As in the case of GLUT1, there are reports of GLUT3 both on neurons (Gerhart et al., 1992) and on cultured glial cells (Sadiq et al., 1990).

In addition, there are reports of the presence of other transporters, GLUT5 and GLUT7 (Simpson et al., 1994), and GLUT4 (Rayner et al., 1994).

At present, there appear to be no reports of a high density of glucose transporters on the astrocyte endfoot (Simpson et al., 1994).

THE METABOLIC FATE OF GLUCOSE

Most studies of uptake of deoxyglucose in the vertebrate nervous system have been interpreted as showing that glucose is taken up directly by neurons (see the section *Conclusions Concerning the Cellular Localization of Glucose Uptake*). If this were so it might be expected that most glucose would be rapidly oxidized to CO_2 and water. As we will see, this is not the case.

Drone Retina

The elucidation of the cellular localization of the biochemical pathways of energy metabolism has proceeded further in drone retina than in any other preparation and resulted in a clear and testable scheme (Tsacopoulos et al., 1994) (Figure 52-3). The preparation has several advantages, which are reviewed in Coles (1989). The only neurons are the photoreceptors, which contain almost all the mitochondria. They are dependent on aerobic metabolism (Dimitracos and Tsacopoulos, 1985), and their

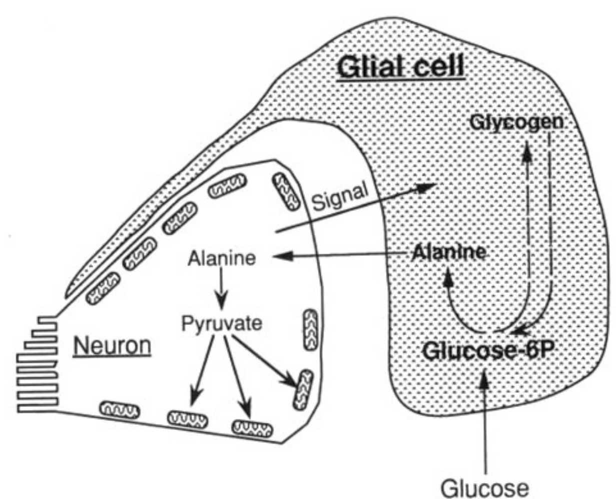
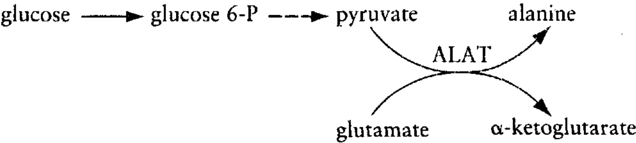


FIG. 52-3. Scheme of major steps in the supply of substrate of energy metabolism to photoreceptor neurons of the drone retina as proposed in Tsacopoulos et al. (1994). The signal from the neurons to the glial cells has not been identified.

oxygen consumption can be increased by light stimulation. As stated in the section about the selective uptake in insect retina, exogenous glucose is taken up selectively by the glial cells so the cellular localization of at least the first reactions in the metabolism of glucose is known. The results, which are summarized here, provide a paradigm to be borne in mind in interpreting results from other species.

The Central Role of Alanine. Glucose is taken up selectively by the glial cells and converted to glucose-6-P (Tsacopoulos et al., 1988). Since glucose-6-P cannot cross the cell membranes, the next reaction must also be in the glial cells. After incubation of a retinal slice for 60 minutes in the presence of glucose whose carbons were uniformly labeled with $^{14}C([U\text{-}^{14}C])$, about half the radioactivity in tissue homogenates appeared in alanine (Tsacopoulos et al., 1994). Other labeled metabolites, in order of decreasing label, were: trehalose, glucose-6-P, glucose-1-P, α-ketoglutarate, glutamate, and a small amount of aspartate. Other amino acids, even those present in high concentrations in the retina, such as taurine and proline, were not detectably labeled, nor was any labeled lactate detected.

Biosynthesis of Alanine in Drone Retina. Tsacopoulos et al. (1994) propose that alanine is formed from glucose by the pathway, ubiquitous in eukaryotes, leading to amination of pyruvate by alanine transaminase (ALAT):



As mentioned above, α-ketoglutarate and glutamate are, after alanine, the two metabolites resulting from multiple reactions that are most heavily labeled after incubation in $[U\text{-}^{14}C]$glucose. The intermediates between glucose-6-P and pyruvate are unlikely to cross cell membranes so the pyruvate is probably formed in the glial cells. Arguments suggesting that the alanine is also formed in the glial cells are (1) that the necessary enzyme, ALAT, is present in freshly isolated glial cells (Veuthey et al., 1994); (2) that if pyruvate, an optimal substrate for mitochondrial respiration, reached the photoreceptors, it would make no apparent sense for it to be transformed into alanine there. Hence, since alanine *is* produced in the tissue, this probably occurs in the glial cells.

The Fate of the Alanine. If alanine were to be a substrate for the photoreceptors it would need to be reconverted to pyruvate for entry into the Krebs' cycle. The necessary enzyme, ALAT, is present in the photoreceptors as well as in the glial cells (Veuthey et al., 1994). Other compounds, notably proline, are involved in the energy metabolism, but the precise pathways have yet to be worked out. Addition of alanine or proline to the superfusate had no detectable effects on the electrophysiology or O_2 consumption in *slices* (nor did glucose, lactate, pyruvate, or trehalose) (Tsacopoulos et al., 1987). Hence, if any of these is an *in vivo* substrate, it must normally be supplied in adequate amounts by the glia.

Other Invertebrates

Treherne (1960) found that glucose injected into the hemolymph of cockroach is almost all converted to the disaccharide trehalose within 30 minutes. Three hours after an injection of $[U\text{-}^{14}C]$glucose into the hemolymph, the radioactive metabolites in the ventral cord were, in order of decreasing quantity, glutamate, glutamine, glycogen, aspartate, and alanine. Note that the incubation time was three times longer than that used by Tsacopoulos et al. (1994).

In the optic and vertical lobes of octopus brain, Cory and Rose (1969) found that after 2 hours incubation in $[U\text{-}^{14}C]$glucose, the amino acids with the highest specific activities were glutamine and alanine.

The Metabolism of Glucose to Amino Acids in Mammalian Brain

Within 20 minutes of injection of $[U\text{-}^{14}C]$glucose into cat or rat, about 70% of the radioactivity in the brain is in amino acids (Vrba, 1962; Gaitonde et al., 1965). At short times (5 and 15 minutes, in mouse) alanine has the highest specific activity (Shimada et al., 1973). The mean free alanine concentration in mouse brain (calculated from Shimada et al., 1973) is 0.75 mmol/L of cell water, but there are marked regional variations. This is low compared to the concentrations of some other amino acids, such as glutamate + glutamine (21.5 mmol/L of cell water). Hence alanine is both synthesized and metabolized rapidly.

When $[^{15}N]$glutamate is added to the medium bathing astrocytes cultured from rat forebrain, alanine is the most heavily labeled metabolite (Yudkoff et al., 1986). This implies the amination of pyruvate in glial cells, as in drone retinal glial cells.

It should be noted that heavy labeling of a compound does not necessarily mean that it is an intermediate in a pathway. Exchange of label could occur

if forward and backward reactions ran with equal velocities without any net flow of atoms (see Siesjö, 1978, p. 103). However, this appears not to be the case for alanine as Yudkoff et al. (1986) have shown that, whereas there was a net uptake of most amino acids by cultured astrocytes, there was a net release of alanine, which implies that there was a net synthesis.

Differences Between Species

Compared to drone retina, the compartmentation of energy metabolism between neurons and glial cells in mammalian brain is less marked, since mitochondria are quite numerous in astrocytes as well as neurons. Although lactate appears to play little role in the metabolism of drone retina it is clearly more important in mammalian brain, and if substrate transfer from glial cells to neurons exists, then lactate may be a major participant (see also the section *Brain Slices* below and Chapters 13 and 32).

EXOGENOUS SUBSTRATES THAT WILL SUPPORT NEURONAL ENERGY METABOLISM

Invertebrates

Leech Ganglion. Freshly isolated leech neurons will survive for hours with glucose as the metabolic substrate (Wolfe and Nicholls, 1967).

Bee Retina. It has been shown that drone photoreceptors take up little, if any, glucose (see the section *In Insect Retina, Glucose Is Selectively Taken Up by the Glial Cells*). Experiments have begun only recently on photoreceptors isolated from their glial cells (Tsacopoulos et al., 1994) and their substrate requirements are not yet defined.

Mammals and Birds

Brain Slices. Schurr et al. (1988) found that synaptic transmission in brain slices failed rapidly when the superfusate contained only 2 mM glucose but was maintained when the glucose was replaced by 2 mM lactate. The synaptic failure may have been due to the inhibitory action of increased extracellular adenosine, due, in turn, to decreased ATP (Fowler, 1993). It has long been known that the brain (of dog) takes up lactate from blood *in vivo* (Himwich and Nahum, 1929).

Cultured Cells. Facci et al. (1985) found that pyruvate, or α-ketoglutarate or oxaloacetate were essential for the survival of chick neurons. These compounds can be involved in the entry of amino acids into the Krebs cycle. In this system, pyruvate was released by astrocytes (Selak et al., 1985).

RELEASE AND UPTAKE OF ALANINE AND LACTATE

If substrates are transferred between cells via the extracellular space, they should be both released from and taken up by tissue.

Bee Retina

After drone retinal slices have been incubated in [$U^{14}C$]glucose, the principal metabolites that appear in the bath are, in order of decreasing quantity of radioactivity: alanine, glutamate, trehalose (Tsacopoulos et al., 1994). Alanine is also taken up by slices. Incubation in ^{14}C-alanine leads to the appearance of $^{14}CO_2$, which can only be formed, in any quantity, in the mitochondria of the photoreceptors, so part, at least, of the uptake is likely to be into the photoreceptors. The concentration of alanine in the interstitial fluid is 31 mmol/L (Cardinaud et al., 1994). This is higher than the mean concentration in the tissue of 14 mmol/L tissue water, so alanine would enter the photoreceptors down a concentration gradient.

Mammals: Alanine and Lactate

Isolated sympathetic ganglia release and take up alanine and lactate, both of which are formed from glucose (Larrabee, 1992). Astrocytes in primary culture release little glucose, but do synthesize lactate and release it (Walz and Mukerji, 1988) in greater amounts than alanine (Dringen, 1992; see also Yudkoff et al., 1986). It is perhaps pertinent that lactate is an effective substrate for brain slices (see the section *Brain Slices*). Changes in extracellular lactate concentration do not simply reflect changes in anaerobic carbohydrate metabolism. Local stimulation of brain tissue causes an increase in local blood flow that exceeds the local increase in oxygen uptake and PO_2 actually increases (Fox et al., 1988; this was also observed by Penfield, 1971). Despite increased availability of oxygen, extracellular lactate concentration also increases (Fellows et al., 1993).

The metabolism of mammalian neuroglial cells is described in detail in Chapter 32.

GLYCOGEN STORES

The conversion of glucose to glycogen allows a large quantity of carbohydrate to be stored in a cell with-

out a harmful increase in osmolarity. The questions that concern us here are whether glycogen is predominantly located in the glial cells, whether it is broken down in response to neuronal needs, and whether a product of the breakdown is transferred to the neurons (see also Chapter 33).

Invertebrate Ganglia

In several species, neuronal perikarya often have invaginations filled by glial fingers, called *Saftkanälchen* by Holmgren (1900). Wigglesworth (1960) showed by histochemistry and light microscopy that in the ganglia of the ventral nerve cord of cockroach, *Periplaneta,* these glial fingers (forming a "trophospongium") were associated with glycogen, but could not tell if the glycogen was in the glial cells or the neurons. The perineurial cells surrounding the ganglion contained much glycogen but there was little in most of the cytoplasm of the outer and inner glial cells that confront the neurons (Figure 52-2B). He also reported that after 3 to 4 weeks of starvation the ganglia had lost almost all their glycogen, but subsequent electron microscopic studies failed to confirm the mobilization of glial glycogen (N. J. Lane, personal communication). In ganglia of the snail, *Planorbis,* and the horse leech, *Haemopis,* glycogen is present in both neurons and glial cells but is generally more concentrated in the latter (Kai-Kai and Pentreath, 1981; Pentreath et al., 1984). In *Haemopis* the glycogen associated with the trophospongium is in the glial fingers, so this is probably also true in *Periplaneta.* Fischer (1968) reported that forcing *Haemopis* to swim to exhaustion caused depletion of glycogen in the glia but not the neurons. However, V. W. Pentreath (personal communication), when he attempted to confirm this, found a quite different result: the glycogen *increased* in the glial cells (and also in the neurons). Hence in these preparations there is no confirmed demonstration of degradation of glial glycogen in response to neuronal need.

Drone Retina: Evidence for Transfer of Substrates of Energy Metabolism from Glial Cell Stores to Neurons

An exceptionally marked compartmentation of energy metabolism in the drone retina between the neurons (photoreceptors) and the glial cells (outer pigment cells) is apparent from the fact that virtually all the mitochondria are in the neurons, while the glial cells are packed with glycogen granules (Perrelet, 1970; Dimitracos and Tsacopoulos, 1985). Since photoreceptor metabolism appears to be obligatorily aerobic, it is possible to calculate the substrate requirements of the photoreceptors from measurements of their oxygen consumption. A retinal slice superfused with a Ringer solution containing no metabolic substrate will function for several hours even when oxygen consumption is increased up to threefold above resting level by light stimulation (Tsacopoulos et al., 1981, 1994; Tsacopoulos and Poitry, 1983). During this time there is a decrease in tissue glycogen content. Within the experimental scatter of the first measurements (Tsacopoulos et al., 1987) this decrease was sufficient to supply the carbon substrate requirements of the mitochondria in the neurons. Recent measurements on larger numbers of retinas show that the decrease in glycogen alone is not sufficient, but also show that there is a decrease in the tissue content of trehalose and proline (Tsacopoulos et al., 1994). Nevertheless, taken together with other results these measurements constitute a convincing demonstration of neurons being supplied with part of the substrates for their energy metabolism from glial glycogen. However, it appears that only a small part of the glycogen stored in the retinal glial cells can usefully be supplied to the neurons. Drones, which live most of their lives in the dark hive, well fed by workers, use their vision only for the short periods, not longer than 40 minutes, when they fly out of the hive (Witherell, 1971). From the figures in Tsacopoulos et al. (1987), stimulation of the photoreceptors for this time would not consume more than about 10% of the retinal glycogen store.

Vertebrates

In vertebrate brain, glycogen is markedly more concentrated in astrocytes than in neurons (Oksche, 1961; Cataldo and Broadwell, 1986). In keeping with this, an antibody against glycogen phosphorylase isozyme B very selectively stained astrocytes and ependymal cells (Pfeiffer et al., 1990; Chapter 33, this volume). Is this glycogen used to provide substrate for neurons? Watanabe and Passonneau (1973) state that "The content of glycogen in the brain remains remarkably constant under a wide variety of conditions, including starvation. . . ." I have not found supporting data in the literature, probably because negative results were not published. Lowry et al. (1964) did show that brain glycogen is depleted within about 10 minutes during ischemia, but "the order of depletion of the compounds of the energy reserve was P-creatine, glucose, ATP, and glycogen, with the rates for the last two being nearly equal." That is, only when energy metabolism was so per-

turbed that [ATP] had begun to fall were the glycogen reserves drawn upon.

In contrast, glycogenolysis can be readily induced by a variety of transmitter substances (see the section *Neural Control of Glial Metabolism* below), although it is not known if products of glycogenolysis are transferred to neurons *in vivo*. However, in mixed cultures of astrocytes and neurons, Swanson and Choi (1993) found that after astrocyte glycogen content had been increased by insulin stimulation the neurons survived longer periods without exogenous glucose.

Conclusion: The Primary Function of Glial Glycogen Is Not to Serve as an Energy Store

Attempts in cockroach, leech, and mammals to induce glycogenolysis in glial cells by starving the animal have not produced confirmed, positive results. In slices of drone retina, neuronal activity does induce glycogen breakdown in the glial cells, but the quantity of glycogen available appears to far exceed any natural requirement by the retinal neurons. See Swanson (1992) for a different view.

NEURAL CONTROL OF GLIAL METABOLISM

There is evidence, in a range of species, that neuronal activity can modify glial metabolism (see also Chapter 32). Since it is the neurons that are active and have obviously increased metabolic needs, this control of glial metabolism, in ways that are not yet understood, may relate to the supply of substrates from the glia. In their classic work on leech ganglion and *Necturus* optic nerve, Kuffler and coworkers found little that affected glial cells apart from changes in extracellular [K^+]. Since K^+ is released into the extracellular space by electrically active neurons, it was an obvious candidate as a signal from neurons to glia (Kuffler and Nicholls, 1966), and the idea that raised extracellular [K^+] modifies glial metabolism has been investigated a number of times.

Invertebrates

Leech and Snail Ganglia. Pentreath and Kai-Kai (1982) found that nerve stimulation increased the uptake of 2DG into ganglia and its incorporation into glycogen, mainly in the glial cells. A similarly increased uptake was observed when the ganglia were incubated in a physiological saline solution in which [K^+] had been increased from its normal value of 4 mM to about 8 mM. Higher [K^+] caused less uptake. Nerve stimulation has been calculated to cause increases in extracellular [K^+] of about 4 mM in leech ganglion (Baylor and Nicholls, 1969). In this preparation K^+ appears to be a strong candidate for a signal, but at least nine neurotransmitters either increase or decrease the quantity of glycogen and multiple controls are therefore more likely (Pennington and Pentreath, 1987).

Drone Retina. Activation of metabolism in the photoreceptors by light stimulation increases the rate of incorporation of glycosyl groups into the glial glycogen both *in vivo* (Evêquoz et al., 1983) and in superfused slices (Evêquoz and Tsacopoulos, 1991). In slices incubated in the presence of 2-deoxyglucose, stimulation of the photoreceptors also increases the rate of formation of 2DG-6P (Brazitikos and Tsacopoulos, 1991). Raised extracellular [K^+], in contrast, appears to have little effect on glial metabolism. Evêquoz and Tsacopoulos (1991) bathed retinal slices in medium containing radioactive glucose and measured incorporation into glycogen. Like Pentreath and Kai-Kai (1982) for the leech, they doubled bath [K^+] but found that in drone retina this produced no significant increase in glycogen radioactivity. Another argument against an increase in extracellular [K^+] as a signal, at least under physiological conditions, is that during the rather uniform light stimulation that the drone retina is likely to experience when flying, extracellular [K^+] appears to rise little, if at all, beyond the value in the dark (Coles and Schneider-Picard, 1989b; Vallet and Coles, 1993). Nevertheless, during continuous stimulation with no increase in extracellular [K^+], there is an ionic response in the glial cells, indicating the presence of a signal, as yet unidentified (Coles and Schneider-Picard, 1989b).

Vertebrates

Raised extracellular [K^+] stimulates glucose uptake in *Necturus* optic nerve (Salem et al., 1975). In mouse cerebral cortical slices [K^+] promotes glycogen hydrolysis that depends on voltage-dependent Ca^{2+} channels (Hof et al., 1988). In addition to this mechanism, astrocytes (like leach glial cells) have receptors for a range of biological substances, including norepinephrine, serotonin, histamine, vasoactive intestinal peptide, and adenosine, all of which can decrease astrocyte glycogen stores, and may be involved in neural control (for references see Chapter 32, this volume; Cambray-Deakin et al., 1988; and Magistretti et al., 1993). In contrast, insulin causes increased formation of glycogen (Dringen and Hamprecht, 1992).

MAIN CONCLUSIONS

1. *There is no evidence in any preparation that glial cells release glucose to supply adjacent neurons.* (In those orders where glial cells form the blood-brain barrier these barrier glial cells may release glucose into the extracellular space of the nervous system, but the neurons are surrounded by other types of glial cell. Further, it is conceivable that if there were gradients of extracellular glucose concentration, then glucose might enter say one end of an astrocyte and leave at the other end; this would have some analogies with spatial buffering of K^+ [Chapter 47], but would be far less efficient because membrane potential would not contribute.)
2. Leech neurons take up glucose that is supplied by the blood to the extracellular spaces. There is evidence that this is also true in mammalian brain, spinal cord, and retina, although in the last case conflicting results have been published. In contrast, in bee retina glucose is obligatorily taken up only by glial cells which supply a different substrate, probably alanine, to the neurons (Figure 52-3). In vertebrate brain, glucose does not need to arrive through the capillaries, since superfused slices with no blood supply can function. *Some features of the metabolism of the mammalian brain might be more readily interpreted if substrates of energy metabolism were normally transferred from glial cells to neurons. Candidate substrates include lactate and alanine.*
3. In none of the preparations studied (ganglia of leech and cockroach, bee retina, mammalian brain) is there confirmed evidence that glycogen in glial stores is degraded *in vivo* to provide metabolic substrate for neurons.

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53 Glial cells and activity-dependent central nervous system plasticity

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PLASTICITY IN THE CENTRAL NERVOUS SYSTEM

Plasticity in the central nervous system has been defined in many different ways. In principle, every change in the physiological properties or morphological appearance of elements in the brain can be subsumed under central nervous system plasticity. This may include developmental sequences, adaptive changes, or responses to central nervous damage. In this chapter the definition will be restricted to two major points. First, it deals only with plasticity relevant to changes in neuronal network properties—areal patterning, axonal circuitry, and functional transmission. This restriction excludes some experimental data summarized in other chapters of this book, for example, development and modifications of the blood-brain-barrier or control of cell lineage (e.g., Chapters 8 and 50). Second, it excludes those plastic changes that reflect genomically controlled developmental sequences, for example, growth or differentiation processes intrinsic to brain constituents. It also excludes the issue of functional regeneration after brain damage and refers instead to other chapters of this book. This chapter focuses on experience-dependent changes in neuronal properties related to development or to learning and memory. Experience- or activity-dependent adaptive changes will be defined as those changes in circuitry, morphology, or synaptic transmission of neurons which occur due to neuronal activity and result in long-lasting functional changes of the neuronal network.

Developmental plasticity and putative mechanisms of learning and memory appear to share a number of features (and possibly also mechanisms). They are therefore dealt with in a more or less parallel and comparative fashion.

A number of processes or phenomena related to the issues under consideration here are discussed in other chapters. Thus, I will restrict the description of such phenomena to the minimum essential for understanding of the models and hypotheses presented. In order to keep this overview brief, only selected examples will be used to support proposed mechanisms of glial involvement in plastic changes. A more detailed summary of experimental data can be found in Müller (1992a).

Developmental Plasticity

One of the most obvious activity-dependent plastic changes in the central nervous system occurs during the development of ordered projections. This includes the elaboration of both long-range projections and local circuitry. This developmental process can be separated into two successive phases: first an activity-independent phase leading to the elaboration of a fairly inaccurate set of neuronal connections, and then a phase of activity-dependent refinement. The first process seems to occur under genomic instruction and relates to neuronal migration, axonal pathfinding, and synaptogenesis (Dodd et al., 1988). The role of glial cells, especially astrocytes, in these processes is described in other chapters of this book (e.g., Chapters 27 and 28). The necessity for complementing this genomically structured patterning of neuronal circuitries by an activity-dependent process becomes clear when comparing the number of synaptic connections (about 10^{12}) to the overall information in the genome and considering the unpredictability and complexity of epigenetic influences on the functioning of the brain. Examples of the latter are the exact positioning of the eyes or corneal curvature, both being critical variables in the correct computation of stereoscopic depth by the central visual pathway. A more general epigenetic factor important for certain human brain structures is the country or area of birth—it is easy to see that this is of considerable importance for language-processing and language-producing structures.

The basic framework for the refinement of neuronal connections appears to follow two general rules:

1. From the preformed connections, the only ones that are stabilized are those that reveal functional effectiveness, as determined by the success in acti-

vating postsynaptic targets. This paradigm is extended by the feature that effective pathways can furthermore increase their local number of transmission sites (synapses) or efficacy (transmitter release or postsynaptic effects). The original hypothesis for this phenomenon has been formulated by Donald Hebb (1949) and was later extended by Gunther Stent (1973).

2. In the vicinity of transmission sites that follow the above-mentioned rule, synaptic sites that do not reveal effective transmission are downregulated (functional depression) or even eliminated (competitive plasticity).

Very often these two mechanisms are restricted to circumscribed periods during development, the so-called "sensitive" or "critical" periods. The temporal onset and the time course of these periods differs from species to species and between different structures. Comparative studies are therefore helpful to assess possible underlying mechanisms. The two mechanisms, that is, stabilization/proliferation of effective and destabilization/elimination of ineffective afferents, are sufficient to explain a number of activity-dependent refinements of neuronal connections. Two examples from the development of the visual system will clarify this point.

With respect to the activity-dependent stabilization of connections, the organization of retinal afferents to their target structures may serve as an example. Retinal afferents to postsynaptic structures are retinotopically organized, that is, neighborhood relations of cells in the retina coding for a given spot in the environment in the central nervous system are maintained in the pattern of synaptic connections in the target regions. This selective organization is gradually developed and refined. Once the afferent fibers have grown into the target and have elaborated a crude topography, visual experience will help to stabilize effective connections and eliminate inappropriately located synapses. This is mediated by the fact that afferents from neurons that terminate in regions where the majority of their "retinal neighbors" make synapses have a high chance to act in concert with their neighbors and activate postsynaptic cells. As these afferents have the opportunity to proliferate they will tend to increase their efficacy and/or synapse number at one given location in the retinotopic organization and thereby increase the chance for further stabilization and increase in efficacy. The activity-dependence of such a process can be shown by deprivation experiments or by suppressing neuronal activity with transmitter receptor antagonists or substances blocking action potentials, for example, tetrodotoxin or glutamate receptor antagonists (Schmidt, 1985). Under these circumstances no refinement of the physiological response specificity occurs.

The second paradigm, the elimination of a given "ineffective" set of afferents when it competes with "effective" afferents, can be clarified by summarizing the effects of monocular deprivation on the thalamocortical projections in the mammalian visual system. Afferents from either eye can convey information from an identical location in the visual environment, that is, are activated by stimuli in the same receptive field. Therefore these afferents compete for the same location in the retinotopic organization in the visual cortex. This normal competition, which usually leads to the spatial segregation of territories innervated by one or the other eye (for review see Müller, 1992a), can be driven into an extreme by depriving one eye from normal vision. Under these conditions the active set of afferents can stabilize and proliferate, while the other is eliminated on the long run because it can never participate in local postsynaptic activation. Again, this process can be shown to rely on neuronal activity as it can be prevented by appropriate pharmacological intervention (e.g., Harris and Stryker, 1986). This paradigm can be used for both the elaboration of local topographies and very global forms of organization, for example, the modality coded in a given sensory structure (auditory information in auditory cortex, visual information in visual cortex, etc.). Possible mechanisms of synapse stabilization and synapse elimination being the very basis for the elaboration of topographical connections will be summarized later.

In summary, developmental plasticity is usually refined to "critical periods" and is based on an activity-dependent selection from a set of exuberant, but imprecisely targeted, afferents by means of stabilization/proliferation and destabilization/elimination. These processes are the basis for the determination of areal boundaries, topographically organized long-range connections, and specific intrinsic connectivities.

Activity-Dependent Plasticity in the Mature System

Activity-dependent plasticity in the mature central nervous system is viewed as an equivalent to the behaviorally defined phenomena of learning and memory. Although the basic mechanisms may often be similar to those underlying developmental plasticity they usually lack a defined "critical period" and are generally believed to rely on changes in synaptic strength (efficacy) rather than resulting from a morphological change in the number/location of synapses. However,

both statements are far from exclusive. For example, it is sometimes hard to decide precisely whether a change in synaptic efficacy is due to a change in the number of transmission sites or due to some change in metabolic processes underlying synaptic strength. This will be clearer when considering hippocampal long-term potentiation as an example for activity-dependent plasticity of a mature system. With respect to the presence of "critical periods," it has to be noticed that efficacy changes may be more dramatic in immature systems than in mature systems (Harris and Teyler, 1984). It may therefore be considered that plasticity in the mature system results from the fact that the critical period for such phenomena is very long (sometimes lifelong).

Hippocampal long-term potentiation is probably the best studied form of activity-dependent plasticity in the mature system and is defined as a long-lasting increase in the efficacy of an afferent pathway to activate a postsynaptic target due to a brief, highly efficient stimulus (for review see Teyler and DiScenna, 1987). Originally this phenomenon was investigated by monitoring the (postsynaptic) field potential elicited by electric stimulation of an afferent pathway. This postsynaptic response is quite constant when the afferent pathway is stimulated at low frequency, for example, every 5 to 10 seconds. One burst of high-frequency stimulation to this pathway—tetanic stimulation with a frequency of 100 Hz for 1 second—results in a long-lasting increase in the efficacy of the afferent pathway to induce a postsynaptic field potential. The increase in the postsynaptic response can either result from the formation of more synaptic sites, or from an increased efficacy in existing synapses. The latter is currently assumed. This conclusion is mainly based on the observation that miniature excitatory postsynaptic currents, which originate from activation of a single synapse, are enhanced following long-term potentiation (Bekkers and Stevens, 1990; Malinow and Tsien, 1990). On the other hand, it has been shown that also the number of synapses is increased following the induction of long-term potentiation (Lee et al., 1980; Chang et al., 1984). It can therefore be assumed that both mechanisms may contribute to the manifestation/maintenance of long-term potentiation, new formation of synapses (similar to the paradigm described for developmental plasticity above) and an increase in synaptic efficacy due to "metabolic" changes. The latter can originate from an increase in transmitter release, a reduced transmitter metabolism/reuptake, an increased receptor sensitivity/availability, or an increase in the efficacy of receptor activation (e.g., increases of ion channel conductance). The analysis of the mechanisms underlying all these possibilities not only includes the final mechanisms proper, but also the intermediate "trigger steps." In the following sections I summarize several ways of how glial cells can contribute to such trigger steps and the proposed possibilities for manifestation of long-term potentiation and its maintenance.

Regeneration

Regeneration can be defined as the recapitulation of developmental processes in the mature system to compensate for central nervous injury. As this issue is discussed in further detail separately in Chapters 27 and 57, I will confine myself to those issues that may be relevant for the understanding of processes underlying normal development and activity-dependent plasticity. As will be outlined later, the widespread failure of regeneration in the mature mammalian central nervous system may shed some light on possible mechanisms underlying critical periods for developmental plasticity. Conversely, mechanisms underlying successful regeneration in certain brain structures or at certain developmental stages may help to understand possible mechanisms of synapse formation in activity-dependent increases of synaptic transmission.

Summary

In the last sections the basic features of activity-dependent plasticity have been outlined. Plasticity in the central nervous system was defined as a change in functional properties of synaptic connections being based on a morphological change (number and location of synaptic contacts) or "metabolic" changes (efficacy of synaptic transmission). Possible underlying mechanisms are summarized in Figure 53-1. With respect to developmental plasticity a crucial feature is the limitation of adaptive changes to restricted critical periods. Plasticity in the mature brain may be based on similar mechanisms but may persist because of a very long critical period. Limitations in the presence or persistence of plasticity over development may be similar to mechanisms that lead to limitations in regeneration. In the following sections I elucidate possible mechanisms of how glial cells can contribute to or even mediate changes in neuronal transmission which result from "experience."

GLIAL CELLS AND DEVELOPMENTAL PLASTICITY

Axon Growth and Areal Boundaries

One basic feature of brain architecture is the presence of functional subunits that are interconnected

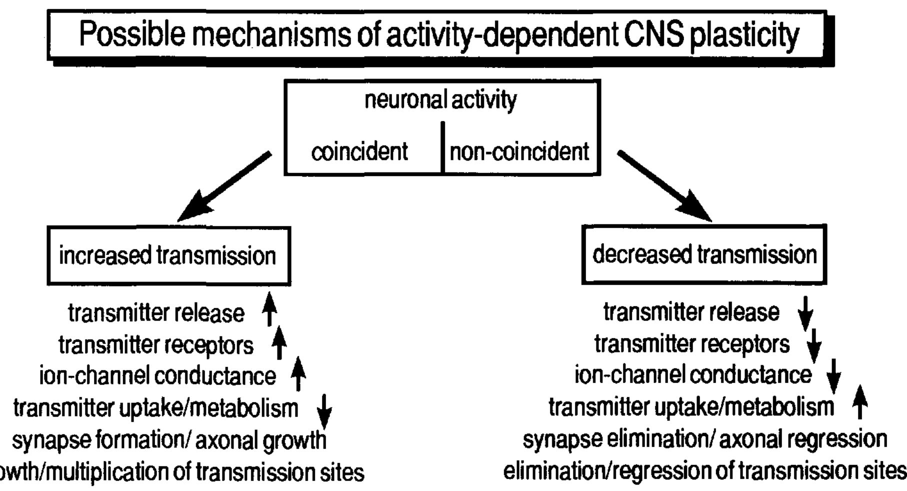

FIG. 53-1. Summary of possible mechanisms underlying activity-dependent plasticity of neuronal transmission. Depending on the correlation of pre- and postsynaptic activity synaptic efficacy can be either enhanced (coincident activity) or reduced (non-coincident activity). Note that the efficacy changes can be mediated by "metabolic" or "morphological" changes. See text for further information.

by specific projections. Neuronal activity has been shown to play an important role in the elaboration of the brains functional architecture. One example for the influence of neuronal activity in the elaboration of functional subunits comes from deprivation experiments. If one sensory modality is impaired, by sensory deprivation or removal of the sensory organs, the according representations are (or remain) functionally innervated by afferents from other modalities (Sur et al., 1990). This phenomenon is based on the principles of axon guidance and activity-dependent refinement of projections. In early development, axons are guided to their future target regions by environmental cues (Dodd et al., 1988). Three possibilities are currently discussed: (1) extracellular guidance molecules, which "mark" selective axon trajectories, (2) spatiotemporal limitations in the accessibility of target structures for innervation, and (3) diffusible molecular "attractors" for selective axonal populations.

To date it is unclear which (or which combination) of these mechanisms underlies axonal targeting. However, recent studies on the retinotectal system of the frog (Gooday, 1990) support the notion that glial cells may be an important component of such mechanisms. When growing axons, *in vitro*, have the opportunity to select between a glial monolayer cultured from tissue of their normal pathway *in situ* and one cultured from a neighboring region (which they do not cross in normal development), they select the former. This finding may indicate that astrocytes carry the information for axonal growth direction. Extracellular membrane proteins or soluble factors originating from glial cells may serve as the guidance molecules. Another guidance cue for outgrowing axons appears to originate from oligodendroglial cells, which express a neurite growth inhibitor. Myelinating oligodendrocytes have been shown to surround growth-permissive "tunnels" for late-growing fiber tracts, thereby contributing to their growth direction (Schwab and Schnell, 1991). As mentioned before, such guidance cues are not sufficient for the elaboration of precise projection patterns. In a following section I address the question of mechanisms for such refinement.

A second phenomenon in early development is the elaboration of area-specific neuronal morphologies and the crystallization of separate structures. The latter can be either areal or intraareal substructures. For both phenomena glial cells seem to be of considerable importance. With respect to neuronal morphology, it has been shown *in vitro* that dopaminergic cells of the mesencephalon elaborate their characteristic morphology only when cultured on a layer of astrocytes or in the presence of media conditioned by astrocytes from the appropriate central nervous system structure (Denis-Donini et al., 1984; Lieth et al., 1989; Rousselet et al., 1990). This effect can not be achieved when using striatal astrocytes. It can therefore be concluded that the glial environment plays an important role in the elaboration of local neuronal specificities. The underlying molecular mechanisms for the glia-neuronal interaction and the characteristics of the obvious regional heterogeneity of glial cells are not understood.

A similar importance of astroglial cells has been shown for the establishment of intraareal structures. A well-studied system is the somatosensory cortex of rodents. This structure is characterized by a highly specific architecture, the so-called "barrels." Barrels are circular arrangements of neuronal somata surrounding a central area containing the den-

drites and receiving the afferent fibers. Each barrel structure receives information of one specific whisker on the rodent's snout (Woolsey and van der Loos, 1970). The formation of barrels depends on the afferent input, and transplantation studies have suggested that the capacity for barrel formation is not confined to future somatosensory cortex (Schlaggar and O'Leary, 1991). One possible mechanism of barrel formation can be deduced from the developmental sequence. Following the ingrowth of afferent fibers, astrocytes initially form the future barrel boundaries. This can be shown by the rapid expression of cytoskeletal proteins at these locations and the expression of specific glycoproteins (Cooper and Steindler, 1986a,b). Only subsequently neuronal somata migrate to or aggregate at these boundaries. Experimental data from another, similar structure support the notion that astrocytes are essential for the formation of barrellike structures. In the developing olfactory system of an insect a very similar sequence of the crystallization of morphological substructures occurs (Tolbert and Orland, 1989). Again, afferent fibers grow into the target, induce glial cells to aggregate at future glomerular structures, which then serve as boundaries for migration of neuronal somata. It can be shown that the glial reaction is an essential step in the formation of glomeruli, as these do not form when the number of glial cells is significantly reduced by glial specific toxins or early irradiation (Tolbert and Orland, 1989).

The data summarized above indicate that glial cells, especially astrocytes, play an important role in the elaboration of areal subdivisions triggered by afferent input and the establishment of axonal connectivities. The underlying mechanisms of neuroglial and glia-neuronal information transfer are not yet understood. The latter seems to be mediated by membrane-bound or soluble glial proteins.

Refinement of Projection Patterns

As summarized in the introduction, mature central nervous circuitries elaborate from a crude projection pattern by the elimination of aberrant and stabilization/proliferation of accurate connectivities. Several observations point to an involvement of glial cells in according mechanisms. Synapse elimination can be mediated by at least three different mechanisms: (1) retraction of synaptic structures/axons, (2) active separation of synaptic boutons from postsynaptic structures and subsequent phagocytosis or degeneration, and (3) destabilization and lysis by environmental enzymes. Glial cells can contribute to the latter two mechanisms. From electron microscopic analyses of the developing spinal cord a sequence of events has been described that corresponds to the second putative mechanism. It could be shown that during early postnatal development of the cat boutons on spinal motoneurons are successively ensheathed by astroglial processes and separated from their postsynaptic target (Conradi et al., 1975). Concomitantly, axonal material was found engulfed in astrocytes. This paradigm would be in line with an involvement of astrocytes in developmentally regulated synapse elimination. Further support for such a causal relation comes from *in vitro* studies. Cultures from cerebellar tissue undergo two phases when cultured for prolonged time periods—an initial exuberant synaptogenesis followed by a period of reduction of synaptic density. When glial cells are eliminated from the cultures the second phase fails to take place (Meshul et al., 1987; Seil et al., 1988). However, substitution of glia by addition of optic nerve astrocytes reinduces the process. Electron microscopic observations have revealed a mechanism similar to the one described in the developing spinal cord. Also in cultured neuromuscular junctions synapse elimination takes only place in the presence of cocultured glial cells (Chapron and Koenig, 1989).

These data point to an important and essential role of astrocytes in activity-dependent synapse elimination. Studies on the developing mammalian visual cortex suggest that only immature astrocytes may mediate synaptic plasticity. The development of astrocytes in the cat visual cortex reveals a close temporal correlation with the critical period for cortical malleability (Müller, 1990, 1992b). Furthermore, transplantation of cultured, immature astrocytes to the mature visual cortex reinduces cortical plasticity restricted to the sites of transplantation (Müller and Best, 1989). Conversely, gliotoxic substances infused into the kitten visual cortex suppress cortical plasticity (Imamura and Mataga, 1993). The exact mechanisms underlying these findings are yet not fully understood, but it is suggested that the effect may correspond to an astroglia-mediated synapse elimination (Müller, 1992a). This may result from active synaptic displacement/phagocytosis, as proposed in the spinal cord and cerebellar cultures. A first step in the activity-dependent synapse elimination by glial cells may be the growth of astroglial processes following neuronal activation (Wenzel et al., 1991). This process may also be mediated by synaptic destabilization/degradation by secreted glial enzymes. *In vitro* studies have shown that astrocytes secrete significant amounts of proteases at time periods corresponding to the height of the crit-

ical period for cortical malleability (Kalderon et al., 1990).

A second glial population presumed to participate in synaptic and axonal remodeling are microglial cells. Transient macrophages, present in the cortical white matter have been shown to be involved in the phagocytosis of exuberant interhemispheric projections in the cat cortex (Innocenti et al., 1983). Recent data from our laboratory show that a similar phenomenon can also be observed at thalamocortical interconnectivities (Müller, in preparation). Up to now there is no evidence for a similar involvement of microglial cells/macrophages in synaptic remodelling of the developing cortex. However, following central nervous damage activated microglial cells have been shown to displace synapses from postsynaptic structures (Blinzinger and Kreutzberg, 1968). This may be indicative for differences in the mechanisms of "physiological" and "pathological" plastic events. From the summarized experiments it can be deduced that glial cells play an important role in the elimination of axons and synapses during development and following central nervous damage. Astrocytes seem to play the dominant role in synapse elimination during development, while microglia is involved in axon elimination during development and synapse displacement following central nervous injury. The mechanism of neuroglial information transfer triggering such events is yet unclear. The presence of such an information transfer is suggested by data from the hippocampal formation and the mammalian cortex, showing that astroglial morphologies and neuronal ensheathement by astrocytes is influenced by neuronal activity (Sirevaag et al., 1991; Wenzel et al., 1991). The signaling pathway may include cations released from active neurons, by neurotransmitters acting on glial receptors, or diffusible second messengers released from active neurons (Müller, 1992). The latter may include novel signaling mechanisms like nitric oxide, which have been shown to target glial biochemical signaling cascades (Agulló et al., 1992). A direct effect of the excitatory neurotransmitter glutamate on filopodia formation in astrocytes has recently been shown (Cornell-Bell et al., 1990) and may represent a first step in the separation of synapses from postsynaptic structures.

Besides their role in synapse elimination glial cells may also be involved in mechanisms of activity-dependent synapse formation. This can be mediated by a release of neurotrophic substances from glial cells or growth-promoting matrix molecules. There is abundant information about the capacity of glial cells to release neurotrophins. A detailed summary of such phenomena and mechanisms can be found in separate chapters of this book (e.g., Chapters 29 and 30).

CHANGES IN SYNAPTIC EFFICACY MAY INCLUDE GLIAL CELLS

Synapse Formation

As summarized above, changes of synaptic efficacy are thought to underlie learning and memory formation. Efficacy changes may originate from multiple mechanisms, some of which can involve glial cells. Efficacy changes due to the formation of additional synapses can be mediated by the mechanisms described for developmental plasticity, that is, the release of neurotrophic factors. Interestingly, growth factors have been shown to support or even induce long-term potentiation in the hippocampus (Terlau et al., 1990a, 1990b). Furthermore, tetanic stimulation has been reported to induce the release of neurotrophins and long-term potentiation-inducing agents (Sastry et al., 1988b; Xie et al., 1991). A more global effect mediated by the release of neurotrophins may explain the finding that long-term potentiation is not restricted to coincidently active pre- and postsynaptic structures, but is also expressed by activated afferent structures in the vicinity (Bonhoeffer et al., 1989).

Influences on Neuronal Excitability

Glial cells can also participate in efficacy changes due to metabolic changes in synaptic transmission. One possibility is an activity-dependent reduction of transmitter uptake by glial cells, which increases the availability of transmitter in the synaptic cleft. Arachidonic acid, which has been proposed as a signaling mechanism from activated neurons to the site of long-term potentiation manifestation (Williams et al., 1989) leads to a prolonged reduction of glutamate uptake into astroglial cells (Barbour et al., 1989). Another possibility for a glial role in activity-dependent efficacy changes is the release of substances by glial cells that affect the postsynaptic sensitivity to transmitters. Astroglia-conditioned media have been shown to increase responses of hippocampal neurons to NMDA-receptor agonists (Forsythe et al., 1988). Glycine, which has been shown to be released from glial cells (Zafra et al., 1990), may be a potential candidate for such an effect. Furthermore, astroglial cells may even release receptor agonists, which may change the excitability of nearby neurons. Homocysteic acid, an endogenous NMDA-receptor agonist, has recently been localized to central nervous astrocytes (Cuénod et al., 1990). Another mechanism for

the modulation of neuronal excitability by glial cells is the change of the extracellular pH (Ransom, 1992). A final possibility is a long-lasting change in the ionic milieu surrounding neurons which may change neuronal excitability.

Direct evidence for a glial participation in synaptic efficacy changes is as yet sparse. One experimental approach used concomitant glial depolarization by intracellular current injection paired with weak- and low-frequency afferent stimulation (Sastry et al., 1988a). It was reported that coincident afferent stimulation and glial depolarization induced long-term potentiation of surrounding hippocampal neurons. The failure to repeat this finding (Konietzko and Müller, unpublished observations) suggests to me that glial depolarization may not be sufficient to induce long-term potentiation in the hippocampus. However, it may be a necessary prerequisite, as blockade of astroglial depolarization by potassium channel blockers prevents long-term potentiation induction (Bonhoeffer and Müller, 1991). It can be hypothesized that glial transmitter receptors have to be activated coincidently with depolarizing signals from postsynaptic structures. Recent data from organotypic hippocampal cultures have shown that stimulation of afferent pathways induces an elevation of the intracellular calcium concentration in astrocytes (Dani et al., 1992). This signaling pathway may furthermore be important for activating possible mechanisms of glial cells that influence neuronal transmission. Recent data have shown that propagating calcium waves in astrocytes can trigger neuronal activity (Needergaard, 1994). Future investigations have to address this issue more specifically.

In summary, a hypothesis of long-term potentiation involving glial cells appears to be attractive, but is speculative. However, the presence of pathways for neuroglial signaling and the multiple possibilities for glial cells to influence neuronal synaptic transmission validate and call for additional studies on this possibility.

POSSIBLE RELATIONSHIP OF MECHANISMS CONTROLLING SUCCESSFUL REGENERATION AND DEVELOPMENTAL PLASTICITY

As detailed summaries on the role of glial cells in the control of successful regeneration can be found in other chapters of this book, I will restrict myself to summarizing the most important findings that can be related to developmental plasticity of neuronal circuitries. Regeneration relies both on the presence of growth-permissive factors in the extracellular environment, as well as on the absence of growth-inhibiting molecules. As detailed elsewhere (e.g., Chapter 57), both conditions rely to a great extent on glial-derived substances. It may be argued that critical periods for developmental plasticity share mechanisms with the control of regeneration, for example, an involvement of glial cells. Support for this hypothesis comes from correlative studies showing that myelination often corresponds to the time of termination of critical periods (Müller, 1992a). During myelination oligodendrocytes have been shown to express neurite growth inhibitors (Caroni et al., 1988; Schwab et al., 1988). Interestingly, it has been reported recently that neuronal activity considerably influences the oligodendroglial cell lineage (Barres and Raff, 1993). It can therefore be assumed that interactions between neurons and oligodendroglial cells determine the time course and the amount of myelination. This may be of considerable importance in explaining the prolongation of critical periods due to sensory deprivation and/or regional differences in the time course of myelination (Müller, 1990). Recent data from our laboratory have shown a correlation of the critical period for visual cortical plasticity and the occurrence of myelin-associated neurite growth inhibitors (Müller et al., 1993). Conversely, glial-derived matrix proteins, which support neurite growth, are predominantly or even exclusively expressed during critical periods. *In vitro* studies have shown that the growth permissiveness of visual cortical tissue reveals a gradual decrease with the time course of the critical period (Müller and Schoop, 1992). Further support for a causal relation of the ability of central nervous structures to perform regeneration and activity-dependent plasticity comes from studies in the olfactory system. These structures reveal a lifelong capacity to regenerate and also reveal a high degree of activity-dependent plasticity (Haberly et al., 1989; Doucette, 1990).

From the forementioned data it follows that glial cells may play an important role in the control of plasticity in the mammalian brain. Especially oligodendrocytes seem to be involved in the restriction of plastic changes in central nervous circuitries to early postnatal periods. Plasticity in structures that have passed the critical period may be reinduced using experimental manipulations which reinduce successful regeneration, namely, an inactivation of oligodendroglial neurite growth inhibitors (Schnell and Schwab, 1990).

In summary, regeneration may underlie similar constraints than critical period plasticity, namely developmental changes of the growth-permissiveness of the tissue. Glial cells may be viewed as the instructor defining temporal and spatial limits of developmental plasticity and regeneration. The mechanisms

Possible glia-neuronal interactions mediating CNS plasticity

Neuron

receptor sensitivity
channel conductance
excitability
transmitter concentration

synapse number
synaptic morphology
receptor number

Glial cell

pH_o↓
K^+
transmitter release
transmitter uptake
growth factors
growth inhibitors
proteases
growth

FIG. 53-2. Glial cells can influence neuronal synaptic efficacy by "metabolic" changes, e.g., influencing neuronal excitability, or "morphological" changes, e.g., synapse formation/regression. See text for further details.

include both temporal constraints in the expression of growth-promoting substances as well as growth-preventing molecules.

SUMMARY

Activity-dependent plasticity plays an important role in the structuring of the central nervous system and allows adaptive changes to occur during development and in the mature system. Two basic mechanisms may explain most if not all plasticity phenomena: (1) morphological changes of the circuitry, that is, synapse formation and synapse elimination, and (2) "metabolic" changes affecting synaptic efficacy, e.g., changes in transmitter availability or neuronal sensitivity to transmitter substances. As shown in the present overview glial cells can easily be implemented in mechanisms underlying such phenomena. Possible mechanisms are summarized in Figure 53-2. Furthermore, several experimental data appear to be more logical when implementing a more global mechanisms of plasticity control than those assumed to act selectively at the synaptic level. As research on a possible role of glial cells in activity-dependent plasticity is a rather recent discipline it is impossible to offer a conclusive view or a fully proven hypothesis. However, the accumulating information on glial cells and their capacity to influence neuronal properties warrants inclusion of this cell population in experimental approaches to and theories of complex brain functions.

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54 Release of neuroactive amino acids from glia

GIULIO LEVI AND VITTORIO GALLO

ASTROCYTES AS RESPONSIVE ELEMENTS IN NEUROTRANSMISSION

During development and in the adult brain, astroglial cells are anatomically situated in close contact to neurons. Astrocytes not only influence information processing between neurons, but are themselves responsive elements in the central nervous system network. In fact, they express voltage- and ligand-gated channels (Barres et al., 1990a; von Blankenfeld and Kettenmann, 1991), as well as receptors coupled to other intracellular second-messenger systems (Murphy and Pearce, 1987; Jensen and Chiu, 1991). These functional properties suggest that astrocytes are a target for signals originating from neuronal activity.

Neurons and astrocytes appear to be part of a loop in which astroglial cells respond to chemical signals from neurons by sending back other messages in the form of neuroactive and neurotrophic substances, which include not only classical neurotransmitters and neuromodulators but also growth factors, cytokines, and prostanoids (for reviews see Merrill, 1991 and Martin, 1992). Most of the experimental studies that revealed such astrocyte features were performed with cultured cells, which may not exhibit the properties of mature, postmitotic astrocytes *in situ*. It is not clear to what extent the properties of astrocytes in the living brain are superimposable on those observed *in vitro*.

It has been long known that astrocytic uptake of neurotransmitters (amino acids and biogenic amines) released from nerve terminals is one of the inactivation mechanisms operating in synaptic transmission (Hösli et al., 1986; Kimelberg, 1986). Due to the absence of storage vesicles, the neurotransmitters taken up by astrocytes are generally rapidly catabolized and are therefore unlikely to be utilized again as chemical signals, as it is believed to be the case for nerve endings. Some transmitter amino acids (glutamate, aspartate, glycine, taurine), however, are present at fairly high concentrations in astrocytes (see Levi and Patrizio, 1992), which have the enzymatic machinery for their synthesis (Hertz, 1982; Huxtable, 1989). Another transmitter amino acid, γ-aminobutyric acid (GABA), appears to be present at minimal levels, but can be synthesized through the putrescine pathway (Barres et al., 1990b; Laschet et al., 1992). In view of the above considerations, it is interesting that recent evidence indicates that certain physiological and physiopathological stimuli can induce release of neuroactive amino acids from astrocytes.

In this chapter, we describe the different types of stimuli that can cause amino acid release, the possible mechanisms of release and alterations of release occurring in conditions mimicking some pathological states of the central nervous system. We will also briefly analyze the behavior of different subpopulations of astrocytes and, therefore, the possibility that these cells are functionally heterogeneous, as suggested also by many other studies (e.g., Shinoda et al., 1989; Usowicz et al., 1989; Wilkin et al., 1990; McCarthy and Salm, 1991; Amundson et al., 1992; Batter et al., 1992; Ito et al., 1992). Finally, we mention some recent studies of the release of glutamate from microglia, a glial cell type that is currently the object of intensive investigation because of its likely involvement in many neuropathological processes. The synthesis and release of neuroactive substances from astrocytes have been reviewed by Martin (1992). The term "release" is used here to mean "exit" from intracellular pools into the extracellular space, independently of the mechanism.

EXPERIMENTAL MODELS USED TO STUDY RELEASE FROM GLIAL CELLS

Because of the intrinsic heterogeneity of nervous tissue, most studies have utilized cell cultures enriched in astrocytes and other glial cell types to analyze the biological properties of these cells independently of other cellular partners present *in vivo*. In particular, the study of the release of neuroactive compounds from astrocytes *in situ* is hampered by the difficulty

of determining the cellular origin of the released material (e.g., astrocytic vs neuronal). An attempt to cope with this difficulty has been made by Paulsen and Fonnum (1989), who used an *in vivo* microdialysis technique to study the release of glutamate and GABA from the rat neostriatum previously treated with the gliotoxin fluorocitrate or with methionine sulfoximine, an inhibitor of the glial enzyme glutamine synthetase. The information provided by this technique, however, is related more to the role of glial cells as a source of transmitter amino acids for neurons than to the process of release from glial cells.

Astrocytes obtained from different brain regions of neonatal animals readily grow in primary culture and can be easily maintained *in vitro* for several days or weeks under conditions that prevent the survival and growth of neurons (Levison and McCarthy, 1991). Often, however, it is necessary to manipulate the cultures to minimize the contamination by oligodendrocytes, their progenitors, and/or microglial cells (McCarthy and DeVellis, 1980; Giulian and Baker, 1986). The purity of the cultures is usually assessed by immunocytochemical staining with antibodies raised against a panel of cell-specific antigens and, in particular, staining for glial fibrillary acidic protein (GFAP), a cellular marker for differentiated astrocytes (Bignami et al., 1972). Primary or secondary cultures are widely used to study the biochemical and physiological properties of a relative pure cell population of astrocytes generally obtained from a single brain area. It has to be noted, however, that studies performed with astrocytes obtained from different parts of the brain have revealed the existence of regional functional heterogeneity, possibly related to the functional specialization of anatomically related neurons (Drejer et al., 1982; Wilkin et al., 1990; Ransom, 1991; Amundson et al., 1992; Batter et al., 1992).

Some experimental studies have dealt with the question of whether astrocyte subtypes differ in their physiological properties (Lerea and McCarthy, 1989; Usowicz et al., 1989; Ito et al., 1992), including stimulus-coupled release of neuroactive substances (Gallo et al., 1989a; Levi and Patrizio, 1992). For this purpose, primary glial cultures have been further manipulated in order to obtain systems enriched in type-2 or in type-1 astrocytes (Aloisi et al., 1988).

Glial cell lines have also been frequently used to study astrocyte physiology and, in particular, release of neuroactive amino acids. For example, the transformed astroglial cell line LRM55 has been extensively used by Martin and coworkers to study taurine release and its mechanisms (Madelian et al., 1985; see below). LRM55 cells express GFAP and exhibit an astrocytic phenotype, including HCO_3^+/Cl^- exchange, glutamine synthesis, morphological changes induced by increasing intracellular cAMP levels and receptor-induced taurine release (Madelian et al., 1985; Martin et al., 1990).

RECEPTOR-INDUCED RELEASE OF NEUROACTIVE AMINO ACIDS FROM ASTROCYTES

Vertebrate glial cells and, in particular, astrocytes express a variety of neurotransmitter receptors, either coupled to membrane ionic channels, or to various intracellular second messenger systems (Murphy and Pearce, 1987; Barres et al., 1990a; Jensen and Chiu, 1991; von Blankenfeld and Kettenmann, 1991). Astrocytic neurotransmitter receptors have been well characterized in their pharmacology and functional properties (Kimelberg, 1988), which appear to be similar in culture and *in situ* (Kimelberg, 1988; Walz and MacVicar, 1988; Clark and Mobbs, 1992). The activation of some receptor types with the proper ligands causes release of various neuroactive compounds from astrocytes (Martin, 1992). The most detailed studies concern the receptor-stimulated release of amino acids (Drejer et al., 1982; Madelian et al., 1985, 1988; Gallo et al., 1989a and 1991; Levi and Patrizio, 1992).

Receptor-Induced Release of Taurine

Martin and coworkers have found that [^{3}H]taurine is released from the cell line LRM55 and from primary cortical astrocytes upon activation of β-adrenergic receptors (Madelian et al., 1985). In LRM55 cells the K_m and the B_{max} for the binding of the β-adrenergic agonist iodohydroxybenzylpindolol were of the same order of magnitude as those found in C6 glioma cells or in primary astrocytes (Madelian et al., 1985). Receptor agonists increased cAMP formation and caused [^{3}H]taurine release with a similar pharmacology. The β-adrenergic agonist isoproterenol was more effective than epinephrine and norepinephrine, and comparable EC_{50} values could be obtained from the dose-response curves of the three agonists on cAMP formation and [^{3}H]taurine release. The antagonists alprenolol and propranolol inhibited the agonist-induced cAMP increase and taurine release in the same concentration range and with similar IC_{50} values (Madelian et al., 1985). The link between cAMP and release of the amino acid was further suggested by the similarity of the time course of the effects on cAMP and taurine release and by the stimulation of [^{3}H]taurine release by the

cAMP analogue dibutyryl-cAMP (Madelian et al., 1985).

The release of [^{3}H]taurine triggered by β-adrenergic receptor activation was not affected by increasing or decreasing the intracellular Ca^{2+} concentration, nor by release of calcium from internal stores (Shain et al., 1989).

Waniewski et al. (1991) investigated whether taurine could be stored in different subcellular compartments and released from distinct pools. The findings in LRM55 glial cells were consistent with the idea that exogenous taurine mixes homogeneously with endogenous taurine and that both endogenous and radioactive taurine are released by isoproterenol from a single intracellular pool. Because the release of five other amino acids (aspartate, glutamate, glutamine, glycine, and alanine) was not affected by isoproterenol, the authors concluded that taurine release is not due to a nonselective loss of cytosolic components and suggested that it may be mediated by the membrane transport system. Other evidence, however, argues against this interpretation (see Martin, 1992, p. 89) and leaves the problem of the mechanism of isoproterenol-evoked taurine efflux unsolved.

Taurine can be released from glial cells also upon activation of different types of receptors (adenosine, serotonin, glutamate, and k-opiate receptors). Madelian et al. (1988) demonstrated that adenosine can increase cAMP accumulation and [^{3}H]taurine release in LRM55 cells, and a pharmacological characterization of the effects observed indicated the involvement of the A_2 subtype of adenosine receptors. Although the adenosine EC_{50} value for taurine release was much lower (1.6×10^{-6} M) than that for cAMP accumulation (5×10^5 M), the analysis of the dose response curve led the authors to suggest that taurine release may be triggered by relatively small increases of cAMP levels. A direct demonstration of the linkage between cAMP elevation and adenosine-induced taurine release is, however, lacking.

Agonists for other receptors were studied in less detail. For example, serotonin and the k-opiate receptor agonist U50-488 were shown to stimulate astroglial taurine release through a cAMP-independent mechanism, but the second messenger possibly associated with this effect is not known (Martin et al., 1988). Interestingly, both serotonin and U50-488 could, at appropriate concentrations, synergize with isoproterenol with respect to taurine release, while either inhibiting (serotonin) or leaving unchanged (U50-488) the cAMP response to isoproterenol. Thus, it seems that more than one intracellular second messenger can be involved in the regulation of taurine release from astrocytes (Martin et al., 1988).

The case of glutamate receptor activation illustrates yet another aspect of evoked taurine release. Ionotropic glutamate receptors of the kainate-AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) subtype are expressed mainly in the type-2 subpopulation of astrocytes (Usowicz et al., 1989; Jensen and Chiu, 1991; Wyllie et al., 1991), while type-1 astrocytes express mainly metabotropic glutamate receptors linked to phosphoinositide turnover (Pearce et al., 1986; de Barry et al., 1991; Jensen and Chiu, 1991). Exposure of type-2 astrocytes to micromolar concentrations of kainate or quisqualate stimulated the release of endogenous taurine (and of other amino acids, see below), while type-1 astrocytes were insensitive to these glutamate agonists (Levi and Patrizio, 1992). The releasing action of kainate and quisqualate was antagonized by 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline (CNQX), a non-*N*-methyl-D-aspartic (NMDA) receptor antagonist inactive on metabotropic receptors. These observations suggest that taurine release was related to the depolarizing action of kainate and quisqualate, which was presumably more pronounced in type-2 astrocytes, due to the much greater expression of the corresponding ionotropic receptors in these cells.

In conclusion (see Figure 54-1, left panel), taurine can be released from astroglial cells upon activation of several types of neurotransmitter and neuropeptide receptors. In some cases the evoked release seems to be associated to an increase of intracellular cAMP concentration, or to other as yet unidentified second messengers, while in other cases release is probably caused by the depolarization consequent to the activation of ionotropic receptors. At least in the latter cases, it is likely that taurine efflux occurs through the Na^+-dependent membrane transport system, whose operation in an outward direction would be facilitated by the increased intracellular Na^+ concentration (see below, for a more detailed discussion).

The activation of glial receptors coupled to taurine release might indirectly cause inhibition of neuronal activity, but *in vivo* evidence to support this hypothesis is lacking.

Receptor-Induced Release of [^{3}H]γ-Aminobutyric Acid

Differently from neurons, glial cells express the non-NMDA subtypes of glutamate receptors, but not NMDA-activated channels (Sontheimer et al., 1988; Gallo et al., 1989a; Usowicz et al., 1989; Berger et al., 1992; Clark and Mobbs, 1992). We first demonstrated that activation of non-NMDA receptors

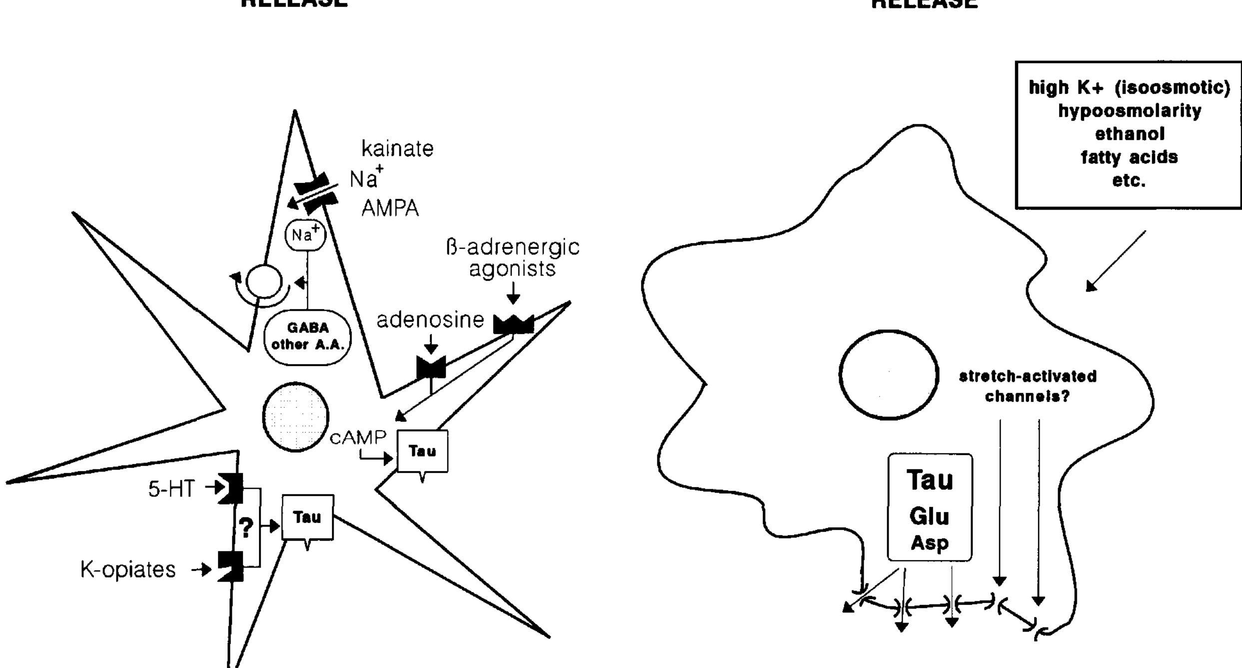

FIG. 54-1. Schematic view of the release of neuroactive amino acids from astrocytes consequent to receptor activation or swelling. See text for details.

by micromolar concentrations of kainate, AMPA and quisqualate stimulated [^{3}H]GABA release from type-2 astrocytes, whereas NMDA was totally ineffective (Gallo et al., 1989a, 1991). The releasing effects of the non-NMDA receptor agonists were inhibited by the antagonists kynurenic acid and CNQX (Gallo et al., 1989a, 1991). In particular, the IC_{50} values for CNQX inhibition of kainate- and quisqualate-stimulated release were in the low micromolar range, similar to those described for neurons (Gallo et al., 1991).

Non-NMDA receptor agonists did not stimulate [^{3}H]GABA release from astrocyte cultures highly enriched in type-1 astrocytes (Gallo et al., 1989a). This was not due to the limited ability of type-1 astrocytes to accumulate [^{3}H]GABA, as compared to type-2 astrocytes (Levi et al., 1983; Johnstone et al., 1986), since the same phenomenon was observed with preaccumulated [^{3}H]D-aspartate (Gallo et al., 1989a), which is avidly taken up by both types of astrocytes (Levi et al., 1983). A more likely explanation (see also below) is that the density of ionotropic non-NMDA receptors in type-1 astrocytes is much lower than in type-2 astrocytes.

Electrophysiological experiments (Usowicz et al., 1989; Wyllie et al., 1991) and fluorescence image analysis of intracellular Ca^{2+} (Jensen and Chiu, 1991) confirmed that glutamate-gated channels of the non-NMDA type are mainly expressed by type-2 astrocytes. In whole-cell patch-clamp recordings, glutamate, kainate, and quisqualate generated large depolarizing currents in type-2 astrocytes. In outside-out patches, single-channel currents induced by glutamate and its agonists displayed five distinct subconductance states identical to those previously identified in cultured central neurons (Cull-Candy and Usowicz, 1989; Usowicz et al., 1989). NMDA did not elicit any response in cerebellar astrocytes.

In the [^{3}H]GABA release experiments, evidence was obtained that glutamate agonists were acting at common receptors with different affinities. In fact, AMPA and quisqualate, applied together with kainate at concentrations around or above their EC_{50}, antagonized the stimulatory effect of kainate on [^{3}H]GABA release. On the other hand, the releasing action of kainate was potentiated by concentrations of quisqualate in the low micromolar range, particularly when the concentration of kainate was at the borderline of effectiveness (Gallo et al., 1989a, 1991). Also in this respect, the behavior of astroglial non-NMDA receptors was similar to that previously described for neurons (Gallo et al., 1989b).

We also addressed the question of the mechanism by which GABA is released in response to glutamate receptor-mediated depolarization. Based on the hypothesis that glutamate receptor-stimulated release

of [^{3}H]GABA could occur through the membrane transport system for GABA, cerebellar astrocyte cultures were preloaded with [^{3}H]GABA and then exposed to the potent GABA uptake inhibitor nipecotic acid for different periods of time, prior to stimulation with kainate. Five minutes of preincubation with nipecotic acid inhibited kainate-stimulated [^{3}H]GABA release by about 50%, whereas exposure to the transport inhibitor for 25 minutes reduced the releasing effect of kainate by 80% (Gallo et al., 1991). Nipecotic acid by itself did not modify [^{3}H]GABA release. In a parallel set of experiments, we also observed that [^{3}H]nipecotic acid was accumulated by type-2 astrocytes and that it could be released by kainate and quisqualate from the same cells (Gallo et al., 1991).

Additional evidence for the involvement of the membrane transport system in [^{3}H]GABA release from astrocytes was provided by experiments in which membrane transport was inhibited by manipulating the ionic composition of the extracellular medium. When the NaCl present in the medium was replaced by LiCl, [^{3}H]GABA uptake into type-2 astrocytes (a strictly Na^+-dependent process) was inhibited (Gallo et al., 1991), but the application of kainate still generated a large non-desensitizing current similar in size and noise level to that produced by kainate in the presence of Na^+ (Wyllie et al., 1991). This is consistent with the fact that Li^+ ions can permeate glutamate-gated channels similarly to Na^+ (Wyllie et al., 1991). Kainate-induced [^{3}H]GABA release was inhibited by about 40% in a medium containing 50% LiCl, and by more than 90% in the medium in which NaCl was totally replaced by LiCl (Gallo et al., 1991). These findings suggest that activation of non-NMDA receptors on type-2 cerebellar astrocytes causes a large influx of Na^+ (or Li^+) ions and that the increased intracellular Na^+ enhances [^{3}H]GABA release by facilitating the operation of the GABA carrier in an outward direction (see Figure 54-1, left panel). However, increased intracellular Li^+ does not support GABA outward transport. Indirectly, these observations also indicate that depolarization *per se* is not sufficient to elicit GABA release from astrocytes. A similar carrier-mediated release mechanism has been described for GABA in retinal horizontal cells (Schwartz, 1987) and for glutamate in retinal Müller cells (Szatkowski et al., 1990). All these findings support the idea that nonvesicular release of amino acids from glia can occur through their membrane carrier, and confirm the old observation that amino acid efflux from neural cells can be mediated by the membrane transport system (Levi et al., 1966).

Interestingly, also in oligodendrocyte/type-2 astrocyte (O-2A) progenitors, that take up [^{3}H]GABA and release it in response to glutamate receptor agonists, the releasing effect of kainate was strongly inhibited when Na^+ was replaced by Li^+ (Gallo et al., 1991).

What could be the functional significance of the [^{3}H]GABA release studies described in this paragraph? Is glutamate likely to stimulate GABA release from astrocytes *in vivo?* The *in vivo* concentration of GABA in astrocytes is probably very low, since these cells show only limited ability to synthesize the amino acid [GABA synthesis from putrescine has been reported to occur *in vitro* preferentially in type-2 astrocytes (Barres et al., 1990b), but also in a cell population presumably enriched in type-1 astrocytes (Laschet et al., 1992)] and the GABA taken up from the extracellular fluid is likely to be rapidly catabolized. Although it cannot be excluded that the activation of glutamate receptors stimulates the release of newly taken up GABA or of endogenously synthesized GABA from type-2 astrocytes, the most cautious way to look at the results reported in this section is to consider the behavior of preaccumulated [^{3}H]GABA as an index of the behavior of other endogenous amino acids (see following section).

Another aspect of the problem of the functional significance of the results described in this paragraph is the expression of ionotropic non-NMDA receptors by astrocytes *in vivo*. As already mentioned, in culture such receptors are present mainly in type-2 astrocytes and O-2A progenitors (see Barres et al., 1990b), although their expression in type-1 astrocytes was noted to increase with culturing time (Wyllie et al., 1991). Glutamate-activated currents were described also in glial cells *in situ*, using the patch-clamp technique in brain slices (Walz and MacVicar, 1988; Berger et al., 1992; Clark and Mobbs, 1992). In the living brain the identification of type-2 astrocytes is still elusive and the exact glial localization of non-NMDA receptors remains to be established.

Receptor-Induced Release of Endogenous Amino Acids

The studies reported above are compatible with the idea that release of neuroactive compounds from astrocytes and their progenitors can be regulated by fluctuations in the extracellular concentration of glutamate. Indeed, endogenous amino acids can be released from cultured astrocytes upon non-NMDA receptor activation. Using secondary cultures of rat neonatal cortex highly enriched in type-1 or type-2 astrocytes, it was found that the endogenous level of most amino acids was substantially higher in type-1

than in type-2 astrocytes. Yet the basal release of only two of these amino acids, glutamine and taurine, was higher in type-1 astrocytes (Levi and Patrizio, 1992). Cultured type-2 astrocytes released several but not all endogenous amino acids in response to kainate or quisqualate. Glutamate release was doubled over baseline and a significant increase in the release of aspartate, glycine, taurine, and alanine was also observed (Levi and Patrizio, 1992). Serine and glutamine were also released by kainate and quisqualate, respectively. The releasing action of the two agonists was reversed by the non-NMDA receptor antagonist CNQX, indicating that release occurred as a consequence of receptor activation. In type-1 astrocyte cultures, neither kainate nor quisqualate modified endogenous amino acid release (Levi and Patrizio, 1992). In the experimental conditions tested (10 minutes exposure to micromolar concentrations of agonists), neither kainate nor quisqualate caused any appreciable increase in cell volume, indicating that the evoked release of amino acids was not related to cell swelling (see below). It is instead likely that the enhanced release of the various endogenous amino acids occurred through a mechanism similar to that described for [^{3}H]GABA in the previous paragraph, namely Na^+-dependent, carrier mediated outward transport.

In rat hippocampal cultures containing mostly type-1 astrocytes, Lehmann and Hansson (1988) found that kainate (100 μM) enhanced glutamine and taurine release by 34 and 85% over baseline, respectively. At a higher dose (1 mM), kainate enhanced the release of all the amino acids measured, glutamate and aspartate being by far the most affected. The effect of kainate was strongly attenuated by the receptor antagonist kynurenic acid, although at millimolar concentrations. Several mechanisms, including cell swelling (see below), could be responsible for the releasing effect of millimolar concentrations of kainate observed in hippocampal astrocytes. However, the possibility of regional differences in excitatory amino acid receptor expression in type-1 astrocytes should also be considered, in view of the regional specialization of astrocytes observed in numerous studies (Drejer et al., 1982; Walz and MacVicar, 1988; Lerea and McCarthy, 1989; Shinoda et al., 1989; Spruce et al., 1990; Wilkin et al., 1990; Amundson et al., 1992; Batter et al., 1992).

DEPOLARIZATION-INDUCED AND SWELLING-INDUCED RELEASE OF AMINO ACIDS FROM ASTROCYTES

Several authors have reported that depolarization of cultured astrocytes with high K^+ stimulates the release of a restricted group of amino acids, namely, taurine (Philibert et al., 1988; Pasantes-Morales and Schousboe, 1989; Martin et al., 1990), glutamate and aspartate (Martin et al., 1990; Levi and Patrizio, 1992), and histidine (Albrecht and Rafalowska, 1987). There is, however, no consensus as to the mechanism by which these amino acids are released, and it is possible that the mechanism of release is not identical for all amino acids and for all glial preparations used. High K^+ is known to cause astroglial swelling when K^+ replaces Na^+ isoosmotically, and hypoosmotic swelling is associated to release of the same group of amino acids (Martin et al., 1990; Pasantes-Morales et al., 1990). On the other hand, exposure of astrocytes to high K^+ in hypertonic conditions depolarized the cell membrane (Walz, 1987), but induced neither swelling nor amino acid release (Martin et al., 1990), suggesting that depolarization is not sufficient to evoke release. At least in the case of taurine, the K^+-induced release has been attributed to the increased tension of the cell membrane due to the augmented cell volume rather than to increased outward transport (for review see Martin, 1992). Increased membrane tension could open stretch-activated ion channels (Kimelberg et al., 1990; Martin et al., 1990) linked to the cell cytoskeleton (Sachs, 1987) and taurine may diffuse through these channels (Figure 54-1, right panel). Indeed, Sanchez-Olea et al. (1991) have shown that in swollen astrocytes taurine fluxes are mediated by passive diffusion.

In the case of glutamate (and possibly aspartate), the astroglial transport system is highly sensitive to extracellular K^+, and elevated extracellular K^+ should facilitate outward transport when the ratio between intra- and extracellular glutamate is high (Martin 1992). In keeping with this expectation, Szatkowski et al. (1990) have elegantly demonstrated, by electrophysiological recordings in salamander retinal Müller cells, that glutamate is released through its membrane carrier upon depolarization. Glutamate transport is electrogenic, therefore glutamate movements across the membrane could be monitored by whole-cell patch-clamp recording of the current associated with activation of the membrane carrier. Elevated extracellular K^+ generated an outward current when Müller cells were patched with pipettes containing both cotransport substrates Na^+ and glutamate (Szatkowski et al., 1990). These experiments, however, do not rule out the possibility that in mammalian astrocytes a component of the K^+-induced glutamate release occurs by mechanisms similar to those hypothesized for taurine.

In contrast to the neuronal release of neurotransmitters, no clear evidence for a direct involvement of

Ca^{2+} in the depolarization-induced and swelling-induced release of amino acids from astrocytes has been provided. The K^+-induced release of taurine was reported to be enhanced (Holopainen et al., 1985) or inhibited (Philibert et al., 1988; Tigges et al., 1990) in Ca^{2+}-free medium containing 1 mM EGTA. Such a medium, however, caused a substantial increase in the spontaneous release of [^{3}H]taurine and [^{3}H]D-aspartate (Holopainen et al., 1985; Philibert et al., 1988; Martin et al., 1989; Holopainen and Kontro, 1990), which makes the results of K^+-evoked release difficult to interpret. Other observations agree in showing that hypotonic, high K^+-, or ethanol-induced amino acid release from astrocytes, believed to be due to swelling, are not Ca^{2+}-dependent (Martin et al., 1989, 1990; Kimelberg et al., 1990, 1993). It is pertinent to recall that exposure of astrocytes to hypotonic media causes a depolarization of the cell membrane proportional to the hypoosmolarity of the incubation medium (Kimelberg and O'Connor, 1988). The fact that Ca^{2+} ionophores are unable to stimulate taurine release from astrocytes (Shain et al., 1989) suggests that an increased intracellular Ca^{2+} level is not sufficient to elicit the release of this amino acid.

Astrocytes have been reported to express voltage-dependent Na^+ channels (Bowman et al., 1984; Barres et al., 1990a). In particular, type-2 astrocytes express the neuronal type of Na^+ channels (Barres et al., 1990b). It may be therefore surprising that veratridine (which depolarizes the cell membrane by locking Na^+ channels in an open position) was unable to elicit release of endogenous amino acids from either type-1 or type-2 astrocytes (Gallo et al., 1982; Levi and Patrizio, 1992). Although depolarization by itself is not expected to evoke amino acid release (see above), the increased intracellular Na^+ level, which should follow the exposure to the alkaloid, would be expected to facilitate outward amino acid transport, similar to that observed in the case of kainate-induced amino acid release (see the section *Receptor-Induced Release of [^{3}H]γ-Aminobutyric Acid*). It is possible that the density of Na^+-channels in the astroglial preparations used was not high enough to warrant a sufficient entry of Na^+ into the cells. At variance with the results summarized above, Holopainen and Kontro (1990) reported that veratridine (0.1 mM) caused a modest, but statistically significant enhancement in the release of preloaded [^{3}H]D-aspartate from cultured cerebellar astrocytes (presumably type-1).

In vivo, astroglial membranes are not believed to be depolarized by the propagation of action potentials. However, in extreme conditions such as epilepsy or spreading depression they could be depolarized by excess extracellular K^+. In view of the *in vitro* observations reported in this paragraph, high extracellular K^+ could elicit the release of some neuroactive amino acids either directly or by inducing astroglial swelling, and this might have functional consequences on neighboring neurons.

GLUTAMATE RELEASE FROM MICROGLIA

Microglial cells are generally believed to play an important role in brain inflammatory, autoimmune, and, possibly, neurodegenerative processes. It has also been suggested that microglia infected with the human immunodeficiency virus (HIV) or exposed to HIV products, such as the coat protein gp120, may produce neurotoxic substances (glutamate, quinolinic acid, or others) responsible for excitotoxic neuron death (Giulian et al., 1990; Gehrman et al., 1992; Lipton, 1992). In a recent study (Piani et al., 1991), it has been reported that microglial cells obtained from neonatal mice produce massive amounts of glutamate, when cultured in an apparently resting state, in a serum-free medium in the presence of 2-mM glutamine. The supernatant of such cultures, collected over a 6-hour period, contained 130 μM glutamate and was highly toxic for cultured cerebellar interneurons, such toxicity being prevented by NMDA receptor antagonists. When microglial cells were activated by exposing them to lipopolysaccharide (LPS) or interferon-γ only a modest increase in neurotoxicity of supernatants was observed (glutamate level, however, was not measured in these conditions). The release of glutamate from microglia is unlikely to be modulated or enhanced by the activation of ionotropic neurotransmitter receptors, which seem to be absent on microglial membranes (Kettenmann et al., 1990).

Although the above observations generated a lot of interest, in view of the possible identification of a microglia-produced neurotoxin, they should be interpreted with caution. Indeed, a massive production of glutamate by resting microglial cells would be hard to reconcile with normal brain function, considering the abundance of these cells in the brain (Lawson et al., 1990). Moreover, there seem to be substantial species difference in the ability of microglia to produce and secrete glutamate, and the magnitude of glutamate release (as evaluated by glutamate accumulation in the culture medium) varies in function of the experimental conditions adopted. In recently published experiments (Patrizio and Levi, 1994) we found that rat and mouse microglia subcultures grown in a serum-free, glutamine-free defined medium, released only minimal amounts of

glutamate over a 6-hour incubation period. In the presence of a physiological concentration of glutamine (0.5 mM), glutamate release was substantially increased. Moreover, mouse microglia was found to contain and release more glutamate than rat microglia. Since glutamate reuptake was virtually absent in microglia, glutamate accumulation in the culture medium increased with time and with cell density. For example, when 10^6 mouse microglial cells were cultured in 0.4 ml of medium in 2-cm^2 wells, the concentration of glutamate in the medium rose to about 60 μM in 6 hours, a value approaching that reported by Piani et al. (1991). A variety of microglia-activating or depolarizing agents, used to simulate pathophysiological conditions [lipopolysaccharide (10 to 100 ng/ml), HIV coat protein gp120 (0.1 to 10 nM), high K^+ (35 mM), or ATP (150 μM)], did not alter rat or mouse microglial glutamate release when the cells were cultured in serum-free medium. However, in the presence of 1% fetal calf serum [which contains protein factors necessary for binding of lipopolysaccharide to its receptor CD14 (Wright et al., 1990)], 100 ng/ml of lipopolysaccharide caused a 2- to 3-fold increase in the accumulation of glutamate in the medium in a 6-hour period. In conclusion, it can be hypothesized that microglia-derived glutamate might contribute to microglial neurotoxicity in those pathological conditions in which intense local recruitment of activated microglial cells takes place. This hypothesis, however, is not supported by *ex vivo* findings showing that the major neurotoxic product of activated microglia obtained from brains subjected to trauma or ischemia can not be identified with glutamate nor other glutamate agonists (Giulian et al., 1993). In conclusion, the issue of glutamate as a putative microglial neurotoxin should be subject to further examination, also in view of the fact that experimental data on human microglia are so far lacking.

PATHOPHYSIOLOGY OF AMINO ACID RELEASE FROM ASTROCYTES

As briefly mentioned in previous sections of this chapter, and amply discussed in recent reviews (Kimelberg et al., 1992; Martin, 1992), astrocyte swelling leads to an enhanced release of some neuroactive amino acids (taurine, glutamate, aspartate). Astrocyte swelling can be induced *in vitro* in a variety of conditions, such as hypoosmotic media, high K^+, high glutamate, ethanol, free fatty acids, lactoacidosis, and acid-base changes, and is known to occur *in vivo* in several pathological states, especially ischemia, trauma, hepatic encephalopathy, status epilepticus, experimental allergic encephalopathy, hypoxia, and brain surgery (for reviews see Kempski et al., 1992; Kimelberg, 1992; Kimelberg et al., 1992). It seems therefore legitimate to suggest that astrocyte swelling can determine an imbalance of extracellular neuroactive amino acids in the brain, which may have important functional consequences.

In other pathophysiological experimental models, the release of some amino acid seems to be affected quite specifically. For example, the neurotoxin aluminum, which has been implicated in the pathogenesis of several neurological diseases, has been reported to enhance the release of [^{3}H]taurine, but not that of [^{3}H]GABA, from cultured rat cortical astrocytes (Albrecht and Noremberg, 1991), while the release of [^{3}H]GABA appeared to be specifically enhanced by high K^+ in bulk-isolated astrocytes obtained from rats with experimental hepatogenic encephalopathy (Albrecht and Rafalowska, 1987). In both these studies, cell swelling was not measured, and only two amino acids were analyzed.

One important and still largely unsolved problem is the involvement of astrocytes in the pathogenesis of brain damage following an ischemic insult. In particular, we are referring to the possible role of astrocytes in the establishment of the high extracellular levels of glutamate that have been reported in the ischemic brain (e.g., Benveniste et al., 1984; Hagberg et al., 1985; Globus et al., 1988; Andiné et al., 1991; Katayama et al., 1991) and have been proposed to be responsible for the generation of ischemic neuronal damage (reviewed by Meldrum, 1989; however, see Globus et al., 1992 for some observations in apparent contrast with the generally held views).

It is difficult to study *in vivo* the cellular origin of the glutamate release induced by ischemia, and this problem has been approached *in vitro* using primary neuronal and astroglial cultures, with conflicting conclusions. Drejer et al. (1985) found that the high K^+-induced release of preloaded [^{3}H]D-aspartate was increased when cerebellar glutamatergic interneurons were exposed to "ischemic" conditions (anoxia + hypoglycemia), but was unaffected when astrocytes were subjected to the same treatment. More recently, Ogata et al. (1992) found that the "spontaneous" release of endogenous glutamate and aspartate was considerably enhanced in astrocyte cultures exposed to a similar "ischemic" condition, whereas the release from neurons was hardly affected. Cell swelling was not measured in either study. Although the two groups reached opposite conclusions, suggesting a major role of neurons (Drejer et al., 1985) or of astrocytes (Ogata et al., 1992), respectively, in the accumulation of extracellular glutamate during ischemia, the two sets of data

are not mutually exclusive. In fact, both the depolarization-induced release from neurons and the spontaneous release from astrocytes could contribute to the establishment of high extracellular glutamate levels. It is obviously difficult to suggest which of the two processes prevails *in vivo*. Since in the living brain extracellular K^+ has been found to be elevated under ischemia (Astrup et al., 1977; Hansen et al., 1980) it is possible that such elevation causes astrocyte swelling, which would lead to an enhancement of glutamate release from these cells, and neuronal depolarization, which would lead to synaptic release of glutamate, if the depolarized neurons are glutamatergic (see Figure 54-2).

CONCLUSIONS

Release of neuroactive amino acids from astrocytes can be induced by "physiological" stimuli, such as neurotransmitters acting at specific recognition sites on the astroglial membrane at "physiological" concentrations, as well as by "pathophysiological" stimuli, such as excess of K^+, excess of glutamate, neurotoxins, altered pH, and so on. Many stimuli of the latter type are secondary to different kinds of brain injury, and stimulate amino acid release through the induction of swelling.

The consequences of neuroactive amino acid release *in vivo* are, at present, only matter of speculation. For example, our knowledge on the existence of regional differences in the expression of neurotransmitter receptors is so far limited (Wilkin et al., 1990), and it is not known whether the release profile or the ability to undergo swelling is identical in astrocytes from different brain areas.

In very general terms, it can be suggested that in physiological conditions the release of neuroactive amino acids from astrocytes induced by the activation of various types of receptors or by small changes in the level of extracellular K^+, consequent to neuronal firing, may represent a further mechanism for finely tuning neuronal activity. It is conceivable that in pathological conditions such a mechanism will be disrupted, and that the establishment of vicious circles will cause self-potentiation of the pathological process. For example, the accumulation of extracellular glutamate and K^+ following an ischemic insult could induce astrocyte swelling with further glutamate and K^+ efflux and consequent potentiation of swelling and excitotoxicity (Figure 54-2).

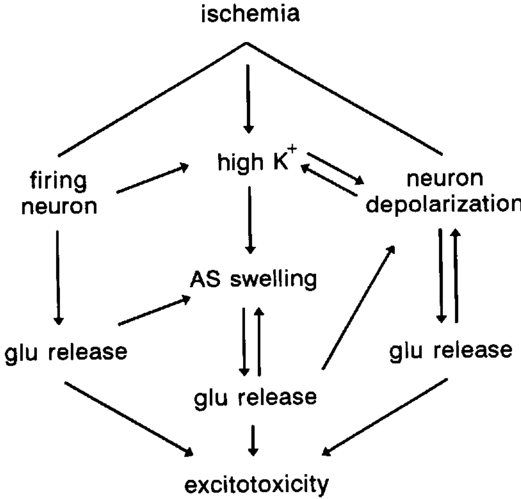

FIG. 54-2. Hypothetical vicious circles in ischemia-related glutamate neurotoxicity. Ischemia may increase glutamate (*glu*) release from firing neurons and cause neuron depolarization, and hence glu release, due to the rise in extracellular K^+. Extracellular K^+ could also cause astrocyte (*AS*) swelling and glutamate loss from these cells. The excess of extracellular glu could potentiate astrocyte swelling and neuron depolarization. The accumulation of extracellular glu resulting from these processes could be neurotoxic.

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XI Role of Glia in Injury and Regeneration

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55 Glial cells and regeneration in the peripheral nervous system

S. A. GILMORE AND T. J. SIMS

In the mid-nineteenth century August Waller carried out a series of investigations which documented that the "elementary" or "primitive" fibers (today designated axons) of a nerve underwent degenerative changes when removed from continuity with the brain or spinal cord. Section of the glossopharyngeal nerve in the frog revealed alterations distal to the site of division as early as the third day postlesion. The substance of these elementary fibers was noted initially to be separated into particles of various sizes, and by the twentieth day these particles were reduced to a granular state. These particles and granules were noted to be contained in what was called a "tubular membrane." Waller (1850) emphasized that such alterations were not unique to the glossopharyngeal nerve but occurred also following section of the hypoglossal nerve and the sciatic nerve. Thus, these early studies established clearly two basic principles of peripheral nerve morphology. First, that continuity of the nerve fibers or axons with a trophic center is necessary for survival. Second, that despite the degenerative changes in the axons, an element of the peripheral nerve associated with these degenerating axons and designated as the "tubular membrane" maintained its integrity. Another key early observation regarding peripheral nerves was that regenerating nerves grew toward distal stumps even when these stumps were misplaced (Ramón y Cajal, 1928). This nonrandom, directed growth was referred to as chemotropism or neurotropism by Ramón y Cajal, who suggested that substances diffused from the distal segment and attracted or directed the regenerating axons. The persistence of Schwann cells and their arrangement into organized "bands" distal to a site of nerve injury was described by von Büngner (1891), and it was these cells which Ramón y Cajal implicated in the production of these tropic substances. In the ensuing years, investigators have documented the potentials for axonal regrowth and defined the roles of other constituents of peripheral nerves, for example, endoneurial tubes, basal laminae, macrophages, and vasculature, in both degenerative and regenerative events in injured peripheral nerves (for reviews see Hall, 1989; Uzman et al., 1989; Fawcett and Keynes, 1990). This chapter focuses on the multifaceted roles of Schwann cells in these events.

SCHWANN CELL PROLIFERATION

During the course of normal growth and development, Schwann cells proliferate to accommodate elongation of growing neurites. Once a state of maturity is attained, proliferation ceases and the population is quiescent (Terry et al., 1974; Brown and Asbury, 1981). This state of quiescence undergoes a rapid transition when a nerve is injured, and within 2 to 3 days Schwann cells in the segment of nerve distal to an injury site revert to a proliferative state (Bradley and Asbury, 1970; Pellegrino et al., 1986). This proliferative state persists until axonal regrowth and myelination or ensheathement are complete.

Mitogens responsible for Schwann cell proliferation are not well understood (Eccleston, 1992). A number of conditions or agents have been demonstrated to be mitogens in the *in vitro* situation, but it is not clear that these play a functional role *in vivo*. Included among these are axonal contact, extracellular matrix, cyclic AMP analogues, and certain peptide growth factors. Of these, loss of axonal contact due to retraction of myelin was considered early (Causey and Palmer, 1953) and remained under investigation in recent years (Crang and Blakemore, 1986). In fact, the maintenance of Schwann cell-axonal association or contact has long been considered to be a major factor in inhibition of significant proliferative activities in the proximal portion of the injured nerve (Ingebrigtsen, 1916; Abercrombie and Johnson, 1942).

Axolemmal preparations and myelin membrane preparations individually exert mitogenic effects on Schwann cells *in vitro*, and the molecules responsible are thought to be membrane-bound polypeptides (Salzer and Bunge, 1980; Salzer et al., 1980). It is

likely that these stimuli come into play in quite different situations *in vivo*, and that different mechanisms are involved. For example, the axolemma exerts its effects directly on the Schwann cell surface and is functional during normal development and in regrowing or regenerative phases. An interesting consideration regarding the axolemmal influence relates to axoplasmic transport. One of the primary functions of fast anterograde axoplasmic transport is the delivery of material for insertion into the axolemma. If fast anterograde axoplasmic transport along a nerve is blocked by focal chilling prior to transection, the premitotic activity of Schwann cells is accelerated (Oaklander and Spencer, 1988). This observation suggests that the proliferation induced in Schwann cells in a segment of nerve in which fast anterograde transport mechanisms are no longer intact may be associated with depletion of a signal normally brought to the membrane by fast transport.

The myelin-derived stimulus as a Schwann cell mitogen, on the other hand, comes into play during Wallerian degeneration. Further, since this stimulus comes into play during a time when myelin is degenerating, the mitogenicity of myelin is considered by some to be dependent upon the presence of macrophages. Under usual circumstances, macrophages enter nerves at the site of injury and become involved with removal of myelin (Beuche and Friede, 1984; Perry et al., 1987; Stoll et al., 1989, and others) along with the Schwann cells (Berner et al., 1973; Nathaniel and Pease, 1963). *In vivo* evidence, on the one hand, indicates that Schwann cells fail to proliferate when a nerve is isolated from macrophages (Beuche and Friede, 1984). Other studies, on the contrary, demonstrate Schwann cell proliferation in explanted segments of nerve lacking macrophages (Crang and Blakemore, 1986). Inability to recruit macrophages into the injured nerve site retards clearance of degenerating myelin but does not preclude nerve regeneration (Lunn et al., 1989). Thus, although the role of macrophages in myelin-derived mitogenicity remains unsettled, it is generally agreed that myelin can serve as a mitogen and that the myelin-derived stimulus requires lysosomal processing by one or more cell types.

The use of myelin-enriched preparations to study Schwann cell proliferation *in vitro* raised the question of whether only those Schwann cells that previously formed myelin are capable of responding to a myelin-derived stimulus. Exposure of Schwann cells to such fractions resulted in proliferation of a portion of the cultured Schwann cells, and continued exposure of the daughter cells to the fraction resulted, in turn, in their proliferation (Yoshino et al., 1987). Evidence from that study, as well as from an earlier study by Salzer and Bunge (1980), suggests that the population of cultured Schwann cells that recognizes the myelin-enriched fraction is the population that previously formed a myelin sheath.

It is not clear if this mechanism exists *in vivo*, but several interesting observations on "myelinated" versus "unmyelinated" nerves merit comment. Schwann cell proliferation is much more robust in myelinated nerves than in unmyelinated nerves. For example, in a myelinated nerve, such as the sciatic nerve, a peak labeling index of 15.5% was noted autoradiographically on the third day following injury (Bradley and Asbury, 1970). A peak labeling index of only 2.5%, however, was noted following injury to the unmyelinated cervical sympathetic trunk (Romine et al., 1976). It has been posited that the increased cell proliferation noted in "myelinated" nerves may be related to the need to fill the space created by the loss of larger axons and their associated myelin coverings or to the participation of Schwann cells in removal of degenerating myelin (Joseph, 1950; Abercrombie and Santler, 1957; Abercrombie et al., 1959; Masurovsky and Bunge, 1971). The enhanced proliferation of Schwann cells in myelinated vs. unmyelinated nerves is considered to play a role in directing regenerating previously unmyelinated axons into the terrain of regenerating myelinating axons, as demonstrated along portions of the vagus nerves (Evans and Murray, 1954; King and Thomas, 1971). Such redirection remains unexplained.

ALTERATIONS IN PHENOTYPIC EXPRESSION

Schwann cells manifest a number of alterations on their cell surfaces and within their cytoplasm in the degenerating/regenerating situation. Intracellularly, intermediate filament protein expression changes, as would be anticipated considering the alterations in cell shape concurrent with Schwann cell activities during proliferative and reparative phases. Two intermediate filament proteins, vimentin and glial fibrillary acidic protein (GFAP), are normally expressed in myelinating and nonmyelinating Schwann cells, respectively. Upon loss of axonal contact, vimentin expression increases in the distal segment and becomes predominant there, while GFAP diminishes. This reverses with axonal regrowth and reestablishment of Schwann cell-axonal contact.

Trophic factor expression also undergoes significant fluctuation, which appears to reflect the status of Schwann cell-axonal contact. The repertoire of these, most of which have been elucidated during

recent years, supports the earlier idea of Ramón y Cajal (1928) that the presence of growth or trophic factors in peripheral nerve plays an important role in regeneration in the peripheral nervous system. The technical capabilities available in the last decade have made it possible to identify some of these factors and to characterize their roles. One of the growth factors associated with peripheral nerve regeneration is nerve growth factor (NGF), a target-derived substance critical to survival of primary sensory neurons, sympathetic ganglion neurons and certain cholinergic neurons of the central nervous system (CNS). The demonstrated capability of the sciatic nerve to release NGF continuously in a culture situation, where it lacked its target organ as a source for this substance, indicated that NGF was synthesized locally in the nerve (Richardson and Ebendal, 1982). This, then, raised the question of whether this phenomenon occurred *in vivo*. To answer this question Heumann and colleagues (1987) examined the levels of NGF and its messenger RNA ($mRNA^{NGF}$) in both intact and transected sciatic nerves of adult rats. They found that levels of NGF in the intact nerve were comparable to those in target tissue of NGF-responsive neurons. The level of $mRNA^{NGF}$ in intact nerve, however, was very low, in contrast to the high level in target tissues, suggesting very little local synthesis in the intact nerve. Following transection, increases of up to 15-fold in $mRNA^{NGF}$ occurred throughout the distal segment, and this increased message level was accompanied by local synthesis of NGF. The proximal segment of the injured nerve manifested changes in NGF synthesis only in its most distal portions, that is, adjacent to the transection site. *In situ* hybridization using a probe to localize the $mRNA^{NGF}$ confirmed its presence along the length of the distal segment and its restriction to the distal end of the proximal segment where neurite outgrowth is to occur. Thus, in response to injury the nerve is capable of synthesizing NGF.

The initial step in the sequence of events by which NGF normally exerts its effects on neurons involves the binding of NGF to specific receptors on the cell surface. The cell surfaces on which these receptors normally occur are surfaces of NGF-responsive neurons. An interesting and important exception to this is the presence of the class II receptors on surfaces of Schwann cells distal to an injury site but not in the intact nerve (Taniuchi et al., 1986, 1988). Immunocytochemical demonstration of the NGF receptor revealed its presence on *all* Schwann cells distal to an injury site and not a localization to Schwann cells associated only with appropriate sensory or autonomic axons. At the ultrastructural level these receptors were localized to the plasmalemma of the Schwann cells. Temporal analysis revealed that the presence of the class II NGF receptors on the Schwann cells was inversely related to axonal contact, that is, that the NGF receptors disappeared from the Schwann cell surface upon reestablishment of axonal contact. Taniuchi and colleagues proposed that these receptors bind the NGF produced by the Schwann cells to their surfaces where it, in turn, can exert both trophic (substrate) and tropic (chemotactic) influences upon the elongating neurites of sensory and sympathetic neurons. Thus, the Schwann cell acts as a temporary source of NGF for the regenerating axon.

The presence of the class II NGF receptors on *all* Schwann cells distal to the site of injury was unanticipated since not all axons in peripheral nerves (cranial or spinal) are NGF-responsive. Injury to a ventral spinal nerve root or to the vagus nerve in the neck, neither of which contains NGF-responsive axons, results also in the appearance of NGF-receptors on Schwann cells distal to the injury. The significance of this is not yet clear.

Another Schwann cell-derived factor upregulated in injured peripheral nerve is glial maturation factor β (GMFβ) (Bosch et al., 1989). Endogenous GMFβ can be demonstrated normally in astroblasts and Schwann cells. Injury (transection or crush) to the sciatic nerve reveals a pattern of expression of GMFβ, which parallels that described for the NGF receptor. In general, GMFβ expression is induced by loss of normal axonal-Schwann cell contact and is repressed by reestablishment of this contact.

The presence of increased trophic factors and/or their receptors in portions of peripheral nerve distal to an injury site is in keeping with the long-standing view of peripheral nerve as a source of trophic substances (Ramón y Cajal, 1928). Within this framework, however, the observation of a decrease in ciliary neurotrophic factor (CNTF) distal to a site of injury (Friedman et al., 1992; Rabinovsky et al., 1992) may appear to be paradoxical. Normal, mature peripheral nerve contains high concentrations of CNTF protein and its mRNA, and Schwann cells are considered as a source of this factor (Rende et al., 1992). Following crush or transection of the sciatic nerve, decreases were found in production of CNTF, in its mRNA level, and in immunoreactivity. Friedman and colleagues (1992) extended their studies of CNTF expression through the regenerative phases and found a reexpression of CNTF protein and its mRNA concurrent with axonal regeneration and myelination, as evidenced in expression of both CNTF and myelin genes. Thus, CNTF appears to be dependent upon the integrity of Schwann cell-axonal

interactions. Obviously, much remains to be elucidated regarding the types and functions of trophic factors produced by Schwann cells.

EXTRACELLULAR MATRIX/BASAL LAMINA

Schwann cells modify their environment by synthesizing and secreting extracellular matrix (ECM) components. These components and other molecules are organized into basal laminae, which surround the mature Schwann cell-axon units (Figure 55-1). Components of ECM can be considered in four categories: collagens; noncollagenous glycoproteins; glycosaminoglycans and proteoglycans. One of the major collagens of the basal laminae of Schwann cells is the type IV collagen which serves as a scaffolding for attachment of other components, *e.g.* fibronectin and laminin. The latter are noncollagenous glycoproteins which mediate interactions between ECM and other cells, and laminin, a component of all basement membranes, is synthesized by Schwann cells. Of the glycosaminoglycans, heparan sulfate proteoglycan is present in the Schwann cell basement membrane.

The formation of the basal lamina is dependent upon interactions between neurites and Schwann cells, and does not require the presence of fibroblasts. This persistence of the basal laminae, however, is independent of the presence of an axon. Intact ECM and its components (laminin and fibronectin) play important roles in neurite elongation during normal development and regeneration (Carbonneto, 1984). Following injury to peripheral nerve or its roots (Figure 55-2), the basal laminae persist as channels within which axons and their myelin degenerate and where Schwann cells proliferate, organize themselves as bands of Bünger and interact with growing neurites. Clearly, the presence of an intact basal lamina appears to be a prerequisite for regeneration in the peripheral nervous system. Components of the ECM in the peripheral nervous system, for example, laminin and certain collagens, are produced by Schwann cells (Bunge and Bunge, 1983; Cornbrooks et al., 1983). Whereas Schwann cell-axonal contact is essential for production of some components of the ECM, for example, laminin (Bunge and Bunge, 1983; Cornbrooks et al., 1983), other components are produced independently of this contact.

A number of roles have been suggested for the components of the ECM. One possible role is that of recruitment and binding of trophic factors so that they can be accessible to the tips of elongating axons. Another important role is that of providing an adhesive substrate upon which the growth cone of the axon can advance. For example, if the filopodia of a growth cone can attach to the substrate, it can then pull the growth cone along with it. If no adhesion occurs, these filopodia may again be incorporated into the growth cone and the latter fails to advance.

Each axon-Schwann cell unit, whether it represents a single "myelinated" axon or a group of ensheathed "unmyelinated" axons, is surrounded by a tube of basal lamina. A nerve, its roots or its branches are filled by these ECM tubes. Once the basal lamina scaffold is formed, it appears that its surfaces—outer (related to mesenchymal elements) vs inner (originally related to plasma membrane of Schwann cell)—are quite different. This consideration arises from observations made when using nerve grafts devoid of Schwann cells. When a segment of sciatic nerve was excised, repeatedly frozen to kill the Schwann cells, and then grafted back into the original site, the regenerating axons were noted to extend along the inner surface but not the outer surface of the basal lamina (Ide et al., 1983). The data derived from that study suggest that living Schwann cells are not as critical for nerve regeneration as is the presence of a basal lamina and that the inner surface of the basal lamina possesses properties that guide and signal neurite elongation. It is well documented that certain components of ECM have specific positions or arrangements (Bunge and Bunge, 1983); thus, it is not unreasonable to speculate that differences in distribution and types of substances on the inner vs. the outer surfaces of the basal lamina may serve to attract or to deter axonal elongation.

Laminin is a major constituent of all basal laminae and its presence in basal lamina of Schwann cells and muscle cells is considered to play a major role in guidance of regenerating neurites (Bignami et al., 1984; Keynes et al., 1984; Madison et al., 1988). Using antibodies against two major components of the Schwann cell basal lamina, laminin and heparan sulfate proteoglycan, it was possible to demonstrate immunohistochemically the persistence and alignment of these ECM molecules in the basal lamina of the distal stumps as long as 28 days following sciatic axotomy. This demonstration of the maintenance of the biochemical integrity is important, since the basal lamina channels act to align the Schwann cells mechanically and also since laminin in association with heparan sulfate proteoglycan promotes neurite elongation (Chiu et al., 1986; Sandrock and Matthew, 1987).

In contrast to the peripheral nervous system, the extracellular matrix of the central nervous system is

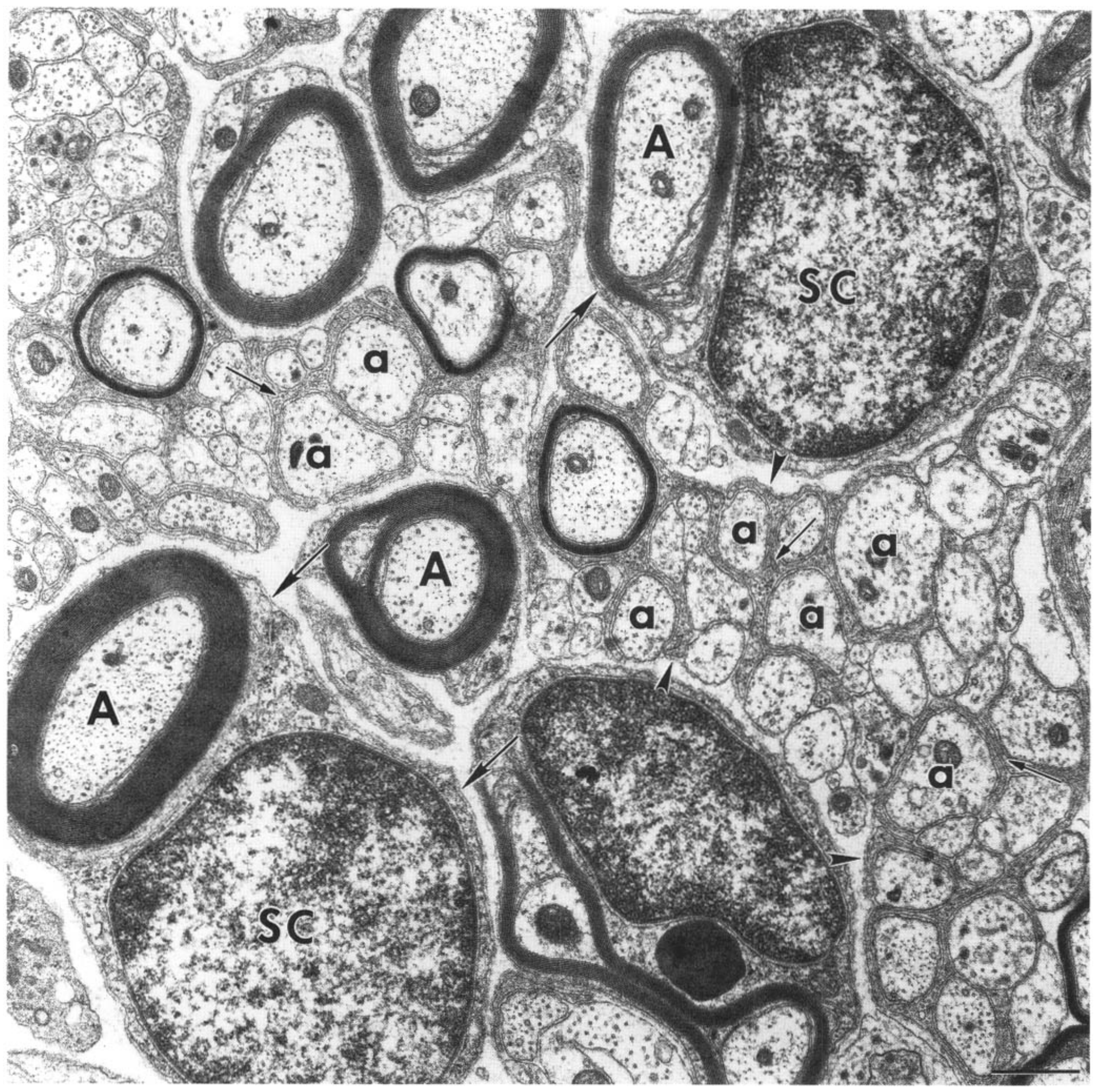

FIG. 55-1. An electron micrograph depicting associations between Schwann cells (*SC*) and axons within a dorsal rootlet of the lumbar spinal cord from a 10-day-old rat. Schwann cell differentiation is fairly advanced by this age, and individual axons exceeding 1.5 μm in diameter (*A*) are generally myelinated by a single Schwann cell, which is covered, in turn, by a basal lamina (*large arrows*). Axons of less than 1.5 μm in diameter (*a*) are generally grouped into bundles by ensheathing Schwann cell processes (*small arrows*). These bundles, in turn, are covered by a thin basal lamina (*arrowheads*). Scale bar = 1.5 μm.

amorphous and virtually lacks collagen and fibrous proteins. This absence of structure within the ECM of the CNS or of proteins responsible for the structured organization of the ECM are thought to contribute to the abortive or limited regenerative capabilities of neurites in the CNS. For example, CNS neurons can respond to laminin as do those of the peripheral nervous system (Liesi, 1985a). The CNS glia, however, do not form laminin continuously as do Schwann cells. It is the continuous expression of laminin that is considered to be critical to growth or elongation of neurites (Liesi, 1985b).

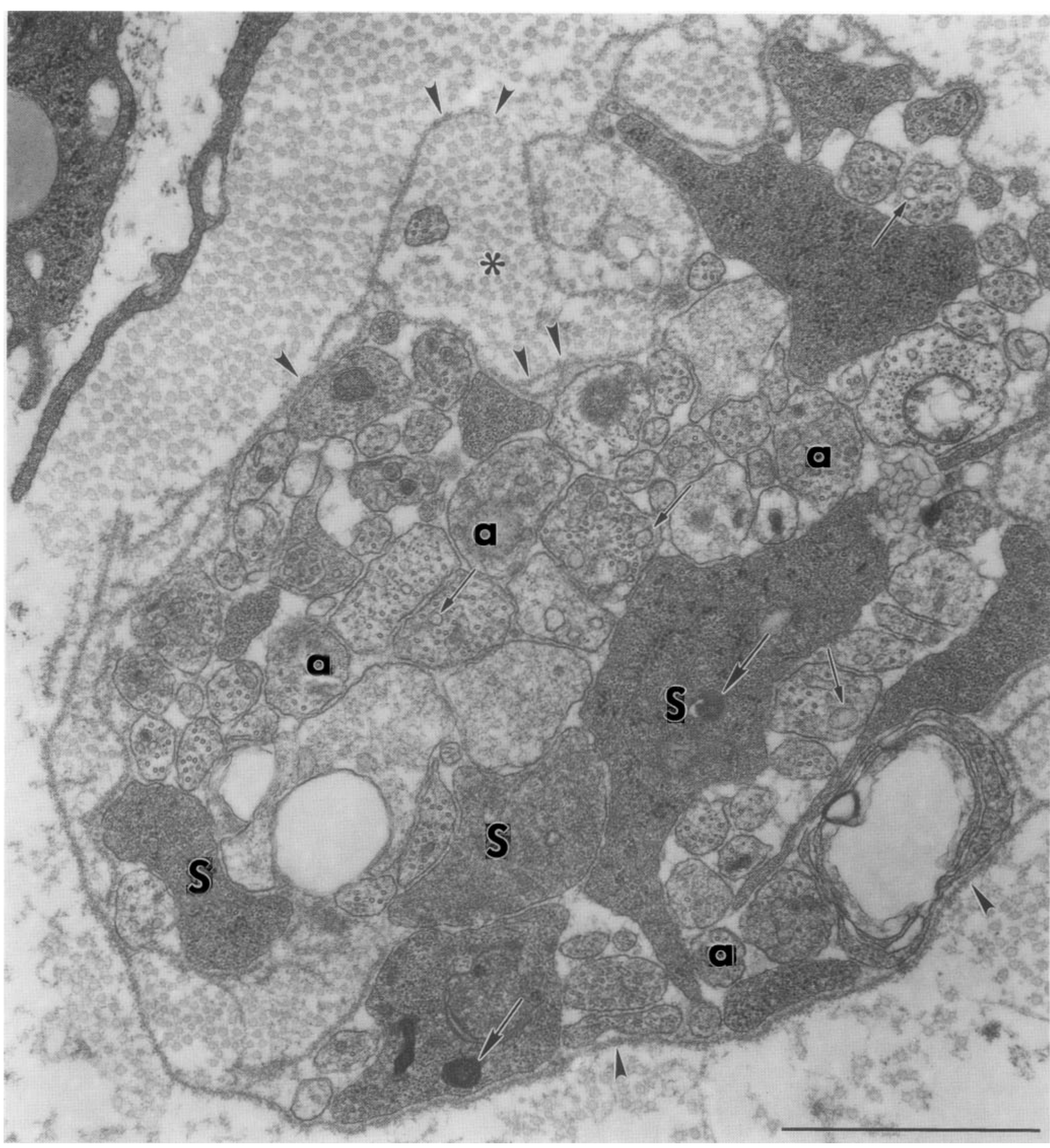

FIG. 55-2. An electron micrograph depicting regenerating axons 5 days following a crush lesion to the sciatic nerve in an adult rat. This sample was taken approximately 3 mm distal to the site of the lesion. Regenerating axons range between 0.08 μm and 2.0 μm in diameter at this time and distance from the lesion; the majority of the regenerating axons in this figure range between 0.1 μm and 1.0 μm in diameter. Regenerating axons (*a*) and Schwann cell processes (*S*) are organized into bundles surrounded, in turn, by a basal lamina (*arrowheads*). Schwann cell processes have a dense cytoplasm and often contain lysosomes (*large arrows*). These regenerating axons often contain microtubules and large pleomorphic vesicles (*small arrows*) reminiscent of growth cone vesicles observed in the developing nervous system. In some areas the basal lamina associated with the bundle forms loops (*double arrowheads*) that incorporate collagen fibers (*). Scale bar = 2.0 μm.

CELL ADHESION

Schwann cells have not only a tropic or attracting influence on elongating neurites, but also play a role in providing appropriate substrates to promote their extension. The laminin-heparan sulfate proteoglycan complex of the basal lamina normally supports growth of neurites, and this function can be inhibited or abolished by the presence of antibodies to this complex (Sandrock and Matthew, 1987). The growth-promoting role of Schwann cells is not solely dependent on basal lamina production, however, and *in vitro* studies have demonstrated that Schwann cells which develop little or no basal lamina in serum-free medium retain their growth-promoting properties (Ard et al., 1987).

The growth-promoting roles of Schwann cells appear to be due, in part, to cell adhesion molecules (CAMs) located on their surfaces. The neural cell adhesion molecule, L1, a surface molecule on postmitotic neurons in the CNS is present also on nonmyelinating Schwann cells in the sciatic nerve of both developing and adult mice (Faissner et al., 1984). Another CAM, the neural cell adhesion molecule (N-CAM), which plays a major role in binding apposing neuronal surfaces, is present also on nonmyelinating Schwann cells (Noble et al., 1985). The binding of neurons to glia is accomplished by a neuron-glia CAM (Ng-CAM), the expression of which is related to migration of neurons along glia and to neurite outgrowth and fasciculation (Thiery et al., 1985). Injury to the sciatic nerve in adult chickens and mice resulted in increased expression of both N-CAM and Ng-CAM in Schwann cells (Daniloff et al., 1986). The timing of their expression postinjury is such as to suggest that these adhesion molecules may be involved in formation of the bands of Bünger and in providing a substrate for neurite elongation. There was a return of these CAMs to normal levels at the time of remyelination. In a study of normal development and injury, the expression of two CAMs, N-CAM, which has been immunolocalized electronmicroscopically to both growth cones and Schwann cells (Martini and Schachner, 1988), and L1 was followed in the mouse (Nieke and Schachner, 1985). During the late embryonic period when most Schwann cells were proliferating, most of these cells expressed both L1 and N-CAM. As myelination progressed through the early postnatal period and into adulthood, expression was reduced and was confined to nonmyelinating Schwann cells. Transection of the sciatic nerve induced a rapid reexpression of both CAMs distal to the injury site, and more than a year was required for the level of the L1 CAM to return to normal.

Although the role of the reexpression of CAMs is not completely understood, they are considered to play important roles in outgrowth of the regenerating axons. Antibodies against N-CAM will inhibit the process of remyelination in regenerating nerves (Rieger et al., 1988). Antibodies against other CAMs, L1/Ng-CAM, N-cadherin, and integrins, applied together but not individually will inhibit growth of axons on Schwann cells *in vitro* (Bixby et al., 1988; Seilheimer and Schachner, 1988). Much remains to be elucidated regarding the roles of CAMs in regeneration *in situ*.

Another protein that may be involved in neurite outgrowth is the S-100 protein, a cytoplasmic protein of both Schwann cells and astrocytes, as well as certain axons. Its level of immunoreactivity is altered in the injured sciatic nerve (Kato and Satoh, 1983; Neuberger and Cornbrooks, 1989; Spreca et al., 1989), undergoing an initial decrease and a later increase. Evidence suggests that the increase in S-100 immunoreactivity in Schwann cells occurs as they become associated with regenerating axons. The role of this protein is not clear, but it is possible that it may be secreted as an extracellular protein and be involved in axonal elongation. Neurite extension properties have been attributed to the disulfide form of S-100β homodimer (Kligman and Marshak, 1985) and Van Eldick and coworkers (1988) suggest that S-100 secreted by CNS glia functions in a paracrine fashion to affect neurite outgrowth. A similar function may be carried out by the Schwann cells of the peripheral nervous system as suggested by Hall (1989).

MYELINATING VERSUS NONMYELINATING SCHWANN CELLS

Assuming that regenerating neurites are successful in traversing an injury site and in entering the distal segment, an important event that remains for reestablishment of structure and function is ensheathement or myelination of these neurites. This raises a basic question: Are there two distinct populations of Schwann cells—myelinating vs nonmyelinating or ensheathing? Or is there a single population capable of expression of either phenotype depending upon appropriate axonal signals? As early as the mid-1900s, cross-anastomosis experiments between myelinated and unmyelinated nerves provided evidence that the axon instructs the Schwann cell as to its need to be myelinated (Simpson and Young, 1945). Those experiments involved anastomoses between a predominantly myelinated thoracic spinal nerve and a predominantly unmyelinated nerve such as ante-

rior mesenteric or splanchnic nerves. Following approximation of the cut ends of these different nerves, axons growing out from the central "myelinated" segment of the thoracic spinal nerve continued to be myelinated in the terrain of the originally "unmyelinated" nerve, for example, the anterior mesenteric nerve, and vice versa. Thus, the parent axon directed the status of myelination. Although these experiments and other earlier studies established that the axon determined its status with regard to myelin formation, they did not provide definitive answers as to whether the distal segments into which the axons regenerated became populated by Schwann cells derived from the proximal segment. In other words, did Schwann cells migrate from the proximal segment into the distal segment? Experiments designed to focus on this question were carried out by Weinberg and Spencer (1976) and Aguayo and colleagues (Aguayo et al., 1976a, 1976b) in the mid-1970s. Their studies employed the cross-anastomosis method, utilizing a predominantly myelinated and a predominantly unmyelinated nerve as in the earlier studies, as well as nerve transplantation techniques. The techniques of tritiated thymidine autoradiography and electron microscopy were employed in these more recent investigations, and the combinations of these methods permitted the determination that significant migrations did not occur and that Schwann cells that previously were of the nonmyelinating or ensheathing type were capable of myelinating under appropriate axonal stimuli. Further, it was postulated that the Schwann cells that undergo division as a result of nerve injury possess a multipotentiality similar to that expressed during normal development. Certainly, the appearance of a number of other characteristics of developing Schwann cells, for example, cell adhesion molecules and surface receptors on Schwann cells at or distal to an injury site, as discussed above, would support this idea. Finally, it should be noted that axons control not only the behavior of Schwann cells with regard to myelin formation but also their survival. Denervated Schwann cells do not persist if the regenerating neurites are prevented from entering the distal segment of an injured nerve (Weinberg and Spencer, 1978).

The differentiation of Schwann cells subsequent to the signaling to be myelinated or to be ensheathed involves a number of changes within the cytoplasm. Since Schwann cell differentiation and maturation involves marked alterations in cellular shape, then alterations in intermediate filament proteins such as vimentin or GFAP are not surprising. Loss of axonal contact due to nerve injury elicits a vimentin-positive, GFAP-negative pattern in Schwann cells distal to the injury site, a pattern noted normally in early development. As Schwann cell differentiation occurs, subsequent to contact with the regenerating neurites, GFAP expression increases (ensheathing or nonmyelinating Schwann cells) and vimentin expression decreases (myelinating Schwann cells) (Neuberger and Cornbrooks, 1989), thereby reestablishing the pattern noted in normal mature nerve.

POTENTIAL ENHANCEMENT OF REGENERATION IN THE CENTRAL NERVOUS SYSTEM

The capacity of the peripheral nervous system to support axonal regeneration serves as the basis for attempts to enhance regeneration in the CNS through use of grafts or transplants of peripheral nerve or Schwann cells. This possibility was suggested early in this century by Ramón y Cajal and the earliest reports of such attempts appear to be those of Tello (1911a, 1911b). Definitive studies, however, depended upon development of such technical capabilities as tracing methods to determine sources of axons observed within a graft and culturing methods to provide sufficient numbers of Schwann cells for transplantation. Recent studies have shown that, when a peripheral nerve graft is placed within the territory of injured CNS axons, the graft can provide a favorable substrate for elongation of CNS axons and can serve as a "bridge" to allow these axons to extend from one region of the CNS to another (for review see Aguayo, 1985; Bray et al., 1987).

The use of peripheral nerve grafts in the CNS has revealed an interesting difference with regard to ability to support CNS axonal growth. To compare the capability of basal lamina tubes alone, in contrast to basal lamina tubes containing viable Schwann cells, to support growth of CNS axons, Smith and Stevenson (1988) implanted these structures into the diencephalon of adult hamsters. The grafts of basal lamina tubes only in which the Schwann cells were destroyed by freezing did not support axonal regrowth as did the graft containing both basal lamina and Schwann cells. This observation stands in contrast to peripheral nerve axons, which were capable of elongating into grafts of basal lamina tubes devoid of Schwann cells (Ide et al., 1983). Whether this reflects a difference between types of neurons remains to be answered.

Purified Schwann cell cultures and the collagen substrata on which they were grown have been grafted into brain (Kromer and Cornbrooks, 1985) or lesioned spinal cord (Paino and Bunge, 1991) in attempts to enhance axonal regeneration. In both sit-

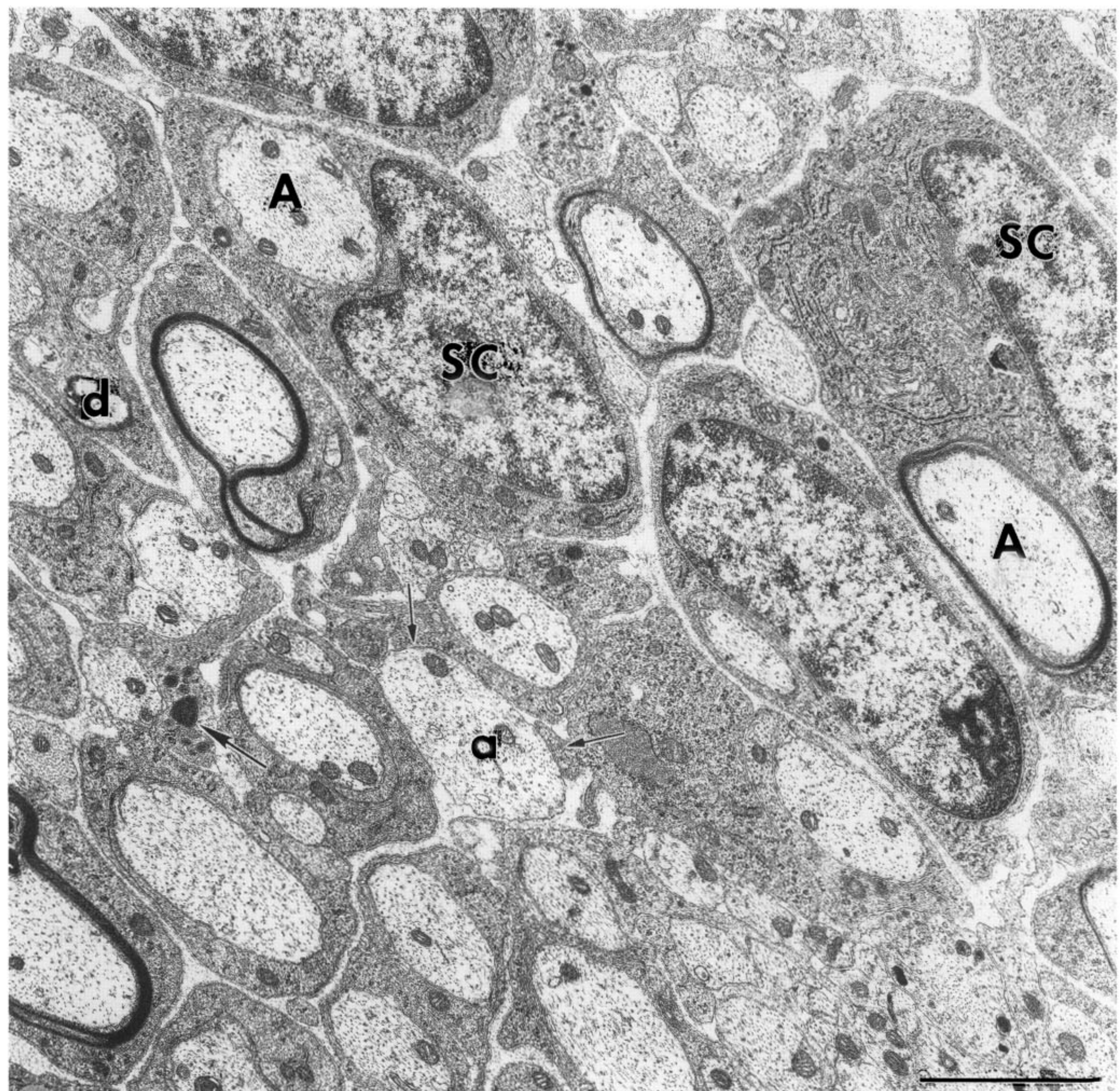

FIG. 55-3. An electron micrograph depicting Schwann cell-myelinated axons in the dorsal funiculus 7 days following injection of cultured Schwann cells into an irradiated portion of the lumbar spinal cord in a 16-day-old-rat. The injected Schwann cells were purified from primary cultures of newborn rat sciatic nerve. Single axons (*A*) with associated Schwann cells (*SC*) are in an early stage of myelination at this time. Some axons of medium diameter (>1.5 μm) are contacted but are not entirely surrounded by Schwann cell processes (*small arrows*). Some degenerative myelin products (*d*), as well as a few lysosomes (*large arrow*), are present in the cytoplasm of the Schwann cells; otherwise, the region occupied by the injected Schwann cells is remarkably free of pathological responses. Scale bar = 2.0 μm.

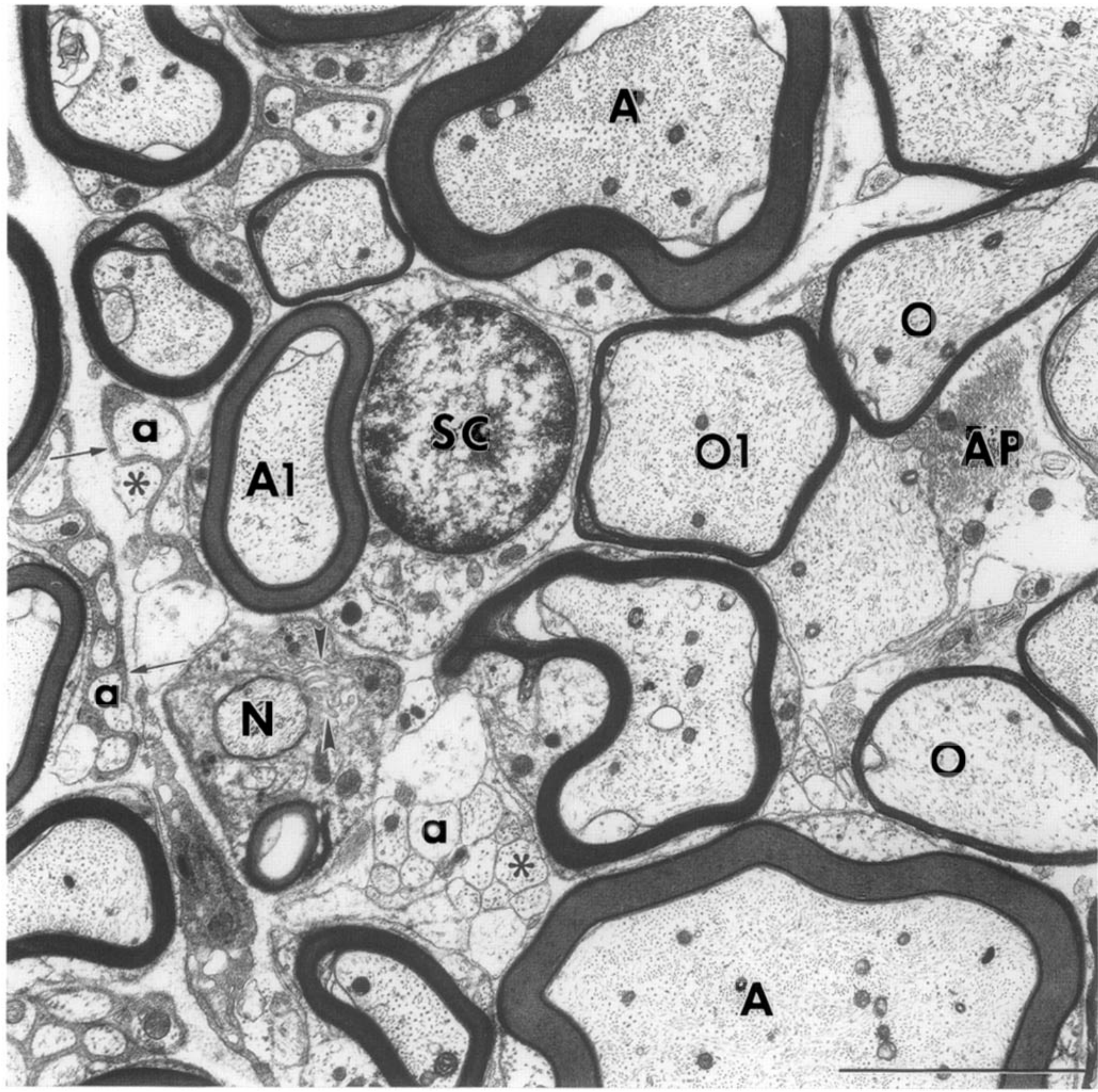

FIG. 55-4. An electron micrograph depicting Schwann cell-myelinated axons in the dorsal funiculus of a rat lumbar spinal cord 90 days following irradiation. As in the peripheral nervous system, intraspinal Schwann cells (*SC*) form sheaths of compact myelin on single axons of large diameter (*A*). Schwann cell-myelinated axons (*A*) are distinguished from oligodendrocyte-myelinated axons (*O*) by the greater thickness of their myelin sheaths on axons of similar diameter; compare axon, *A1*, having a much thicker myelin sheath derived from a Schwann cell with the adjacent axon, *O1*, surrounded by an oligodendrocyte-derived myelin sheath. Small diameter axons (*a*) remain unmyelinated (*) or surrounded by Schwann cell processes (*arrows*). Intraspinal Schwann cell processes (*arrowheads*) contact the nodal region of axons (*N*) in a manner similar to that observed in the peripheral nervous system. Although fewer in frequency than in the normal 90-day-old spinal cord, astrocyte processes (*AP*) are present in these regions of mixed myelin types. Scale bar = 4.0 μm.

uations regenerating axons, ensheathed or myelinated by the Schwann cells, were observed in the graft. Collagen grafts lacking Schwann cells failed to support axonal growth in the lesioned thoracic spinal cord.

Cultured Schwann cells lacking substrates also have been transplanted into the CNS in attempts to repair myelin deficits or enhance axonal elongation through lesion sites (Baron-Van Evercooren et al., 1992; Blakemore and Crang, 1985; Duncan et al., 1981; Martin et al., 1991) (Figure 55-3). Data derived from each of these investigations and from our laboratories (Gilmore and Sims, 1993) indicate that the transplanted Schwann cells support axonal elongation and/or ensheathe or myelinate axons. The types and origins of the axons with which these transplanted Schwann cells establish ensheathing or myelinating relationships is not always clear. Nonetheless, they provide clear evidence that Schwann cells, even those from immortalized lines (Baron-Van Evercooren et al., 1992), survive and form myelin following transplantation into the CNS.

In the foregoing experiments the physical integrity of the CNS was breached or disrupted to allow for

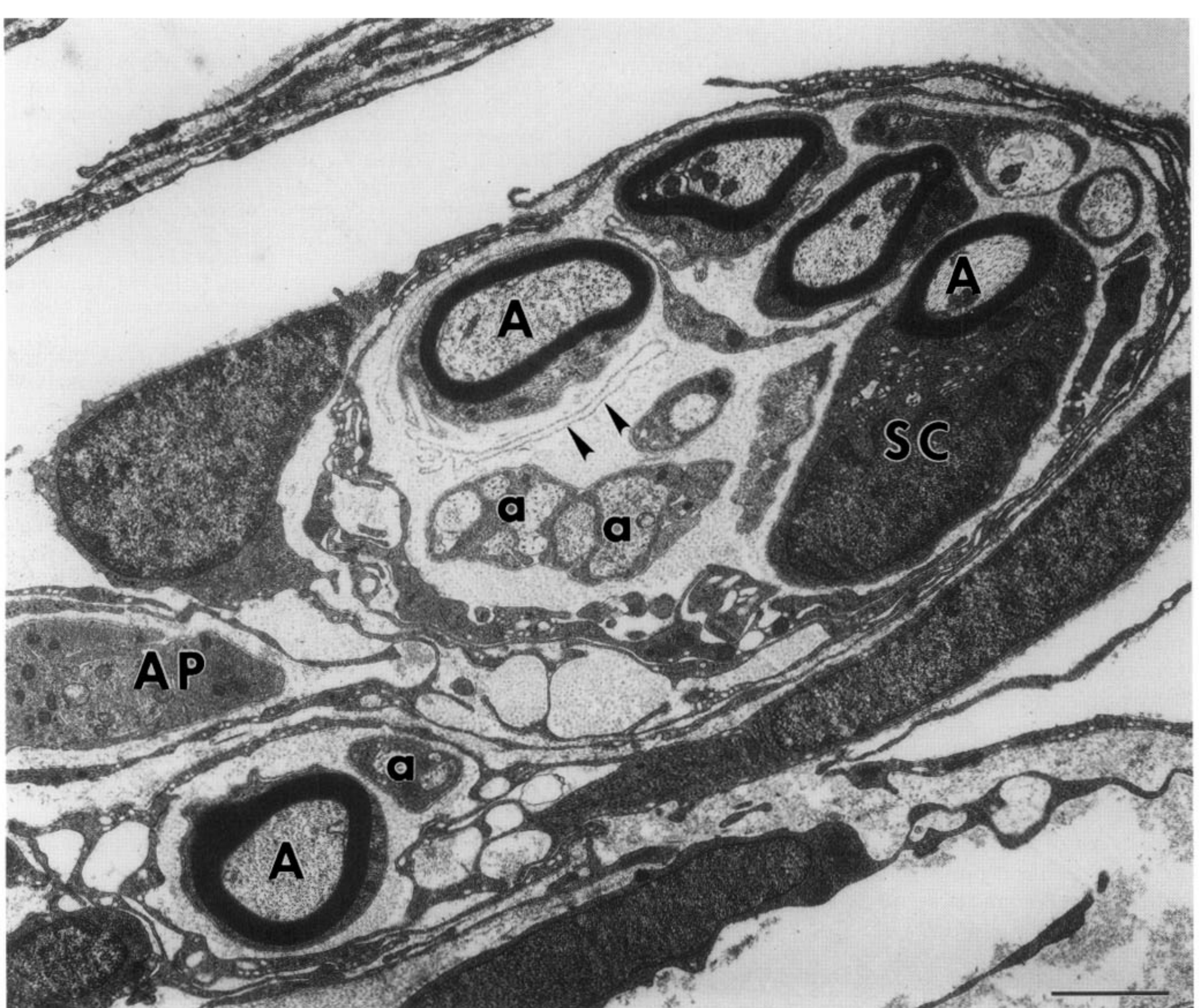

FIG. 55-5. An electron micrograph of regrowing dorsal root axons near the transition zone between central nervous system and peripheral nervous system, from an irradiated rat in which the dorsal root was lesioned 60 days earlier. Regrowing axons are associated with Schwann cells (*SC*) that form compact myelin on larger axons (*A*) or ensheathe axons of smaller diameter (*a*). An empty basal lamina tube remains within the fascicle (*arrowheads*). A small group of astrocyte processes (*AP*) is present, but these are markedly fewer than observed in a nonirradiated, root-lesioned rat in which this region consists principally of reactive astrocyte processes. Scale bar = 2.0 μm.

the placement of the cells, grafts or transplants. This physical disruption activates a series of responses in the astrocyte population and in the microvasculature, which, in turn, complicates evaluation of the behavior and potentials of Schwann cells within the CNS environment. A model in which Schwann cells can be induced to develop within the confines of the CNS in a predictable, reproducible fashion, has been developed by Gilmore and colleagues (Gilmore and Duncan, 1968; Gilmore and Sims, 1986). This model involves depletion of the glial population in the spinal cord of the immature rat by exposure to ionizing radiation. Subsequently, Schwann cells develop within the spinal cord, particularly in the dorsal funiculi, where they are capable of proliferating (Gilmore, 1971; Gilmore et al., 1982; Gilmore and Sims, 1986) and of myelinating or ensheathing axons (Gilmore and Duncan, 1968; Gilmore et al., 1982; Gilmore and Sims, 1986; Sims and Gilmore, 1989) (Figure 55-4), which under normal circumstances would be myelinated by oligodendrocytes. Given the predictability of this induction, the model has proven useful in evaluating many aspects of Schwann cell behavior in the CNS environment, including axolemmal/Schwann cell interactions (Black et al., 1986; Sims and Gilmore, 1989). Additionally, recent investigations by the authors (Sims and Gilmore, 1994) demonstrate that the presence of the radiation-induced Schwann cells in the dorsal root entry zone facilitates regrowth of lesioned dorsal root axons from the peripheral nervous system terrain of the dorsal root into the CNS terrain of the spinal cord (Figure 55-5).

Taken together, one important implication of the studies cited above based on paradigms ranging from grafting of segments of whole nerve to transplanting of cultured Schwann cells to other means, for example, radiation, of induction of Schwann cells within the CNS is that Schwann cells within the CNS environment possess many of the characteristics of Schwann cells in their normal peripheral nervous system domain. Their ability to sustain regrowth of injured axons and redirect them into their usual sites of distribution in some areas of the CNS provides fertile ground for further examination of Schwann cell capacities and interactions within the CNS milieu.

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56 Gliosis

AMICO BIGNAMI AND DORIS DAHL

Bundles of 10-nm (intermediate) filaments in the astrocyte cytoplasm are the electron microscopic equivalent of glial fibers at the light microscopic level. Accumulation of glial fibers is the histological landmark of the astrocyte response to injury, appropriately named fibrous gliosis (Figure 56-1). These fibers fill up the space resulting from the loss of myelin and neurons.

Glial fibers first led to the discovery of neuroglia as a substance that appeared to be brain-specific and thus different from connective tissue but still distinct from nerve. In 1846, Rudolph Virchow, a German pathologist, identified under the ependymal lining of the lateral ventricles, "a structureless membrane, which often appeared to be formed by very fine and pale fibrils lying parallel to each other in a rather regular pattern" (Weigert, 1895). For several years Virchow thought he could dissect this membrane with a scalpel, but he later came to the conclusion that it extended without boundaries through the substance of the brain, forming a sort of connective tissue holding nerve together (hence the name neuroglia, which means nerve glue). Virchow was also the first to describe a pathological overgrowth of the substance he had discovered in the spinal cord of a patient with tabes, a form of neurosyphilis resulting in the degeneration of the dorsal columns. After hardening with chromic acid, a fine mesh of fibers had become apparent. Under these conditions, normal spinal cord tissue has a fine granular appearance probably due to the presence of birefringent myelin sheaths. In view of the techniques available to Virchow, one may understand Deiter's statement that the discovery of neuroglia appeared to be the result of divination rather than demonstrable facts (Weigert, 1895).

The next major step was the development by Weigert of a method allowing the selective staining of glial fibers (Weigert, 1895). The selectivity of the method for neuroglial fibers led Weigert to believe that they were made of a specific substance. This was an important factor for initiating studies aimed at demonstrating the specificity of the protein forming the glial fibers, that is, glial fibrillary acidic protein (GFAP). Furthermore, Weigert's illustrations provided the reference for assessing the specificity of GFAP antibodies (Bignami and Dahl, 1974a).

Excellent reviews of gliosis have been published (Reier et al., 1983; Lindsay, 1986; Reier, 1986; Malhotra et al., 1990; Hatten et al., 1991). Our main claim to originality in this chapter is that we do not view recent developments from the perspective of conventional neuropathology. The factors that trigger the astrocyte response to injury as well as the role of the reactive astrocyte in inflammation will not be discussed here (see Chapters 44, 45, and 46 in this volume).

PRIMARY AND SECONDARY GLIOSIS

Gliosis occurring in several neuropathological conditions, particularly gliosis associated with epilepsy (Ammon horn sclerosis) was believed in the past to result from a primary disease of the glia and thus to be kept distinct from gliosis resulting from destruction of nerve tissue, that is, secondary gliosis (Obersteiner, 1912). It is now believed that gliosis is almost invariably a secondary phenomenon, although not necessarily the result of neuronal degeneration. It is also believed that the glial scar may play a role in pathological events, such as the absence of functional regeneration in the central nervous system and epileptogenesis. Since the role of glia in regeneration is covered in Chapters 57 and 60, we confine our discussion to epileptogenesis.

Uptake of potassium and neurotransmitters are important glial functions (see Chapters 47 and 48). It is thus conceivable that impairment of these functions in a glial scar may result in the formation of an epileptic focus. In fact, an impairment of potassium homeostasis was proposed as a factor in the development of posttraumatic focal epilepsy (Pollen and Trachtenberg, 1970). However, no differences were observed between normal and gliotic cortex in the diffusion coefficient for potassium (Pedley et al., 1976; Dietzel and Heinemann, 1986). Furthermore, experimental elevation of the potassium concentration in damaged cerebral cortex did not result in seizures.

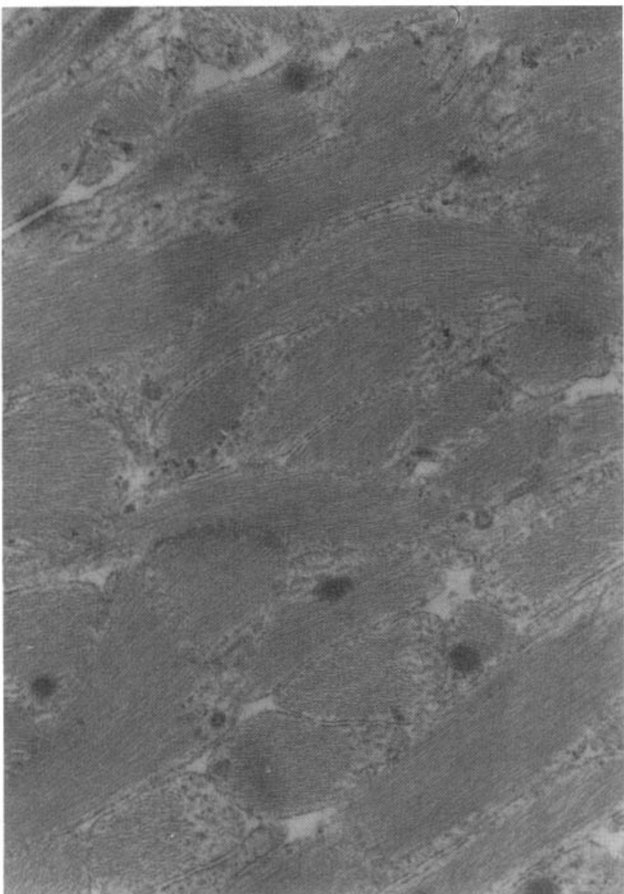


FIG. 56-1. Electron micrograph of the severely gliosed hypothalamus in a sheep with natural scrapie, a slow viral infection. Astrocyte processes are packed with intermediate filaments. The bundles of filaments appear as glial fibers by light microscopy. Gliosis is a prominent finding in slow virus infections. Antibodies raised to scrapie-infected brain selectively stained astrocytes by indirect immunofluorescence, thus suggesting that the agent was localized in these cells. However, the activity could be adsorbed by normal brain extracts (Gajdusek, personal communication). Compared to other brain proteins, glial fibrillary acidic protein (GFAP) is a good antigen and inoculation with human brain extracts may result in the production of GFAP antibodies. [From Dahl and Bignami (1973a), with permission.]

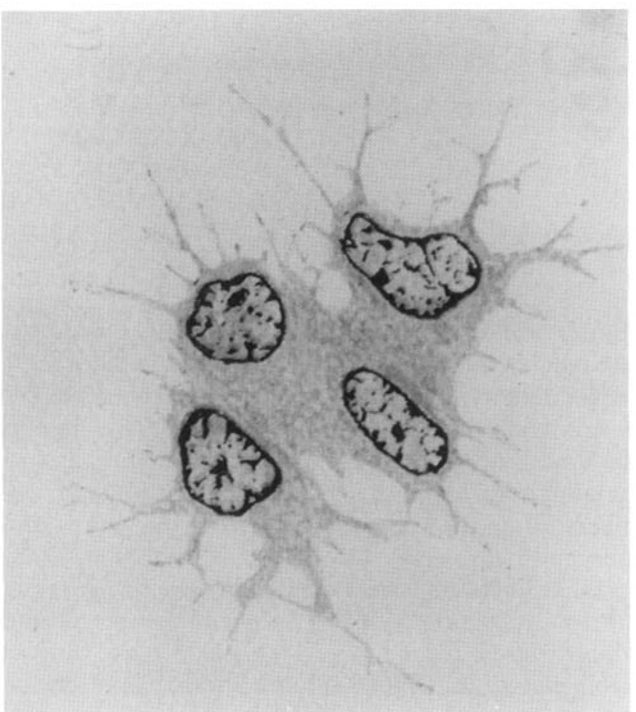


FIG. 56-2. *Gliarasen.* Figures like this first suggested that astrocytes are capable of dividing. In our experience, *Gliarasen* are numerous in subacute measles encephalitis where they may represent the end result of neuronal destruction (see Chapter 59 in this volume for the astrocyte response to selective neuronal necrosis). Nissl preparation. [From Spielmeyer (1922).]

A recent study of astrocyte cultures derived from epileptic foci suggests that neuronal excitability could result from abnormalities in the handling of glutamate (Magge et al., 1992). In these cultures, glutamate synthesis and uptake were markedly enhanced compared to cultures derived from normal brain.

A possible exception to the general rule that gliosis is a secondary phenomenon is represented by Huntington's disease. Lesions comparable to those observed in Huntington's disease, that is, loss of small neurons in the neostriatum and gliosis, can be experimentally produced by the nigrostriatal injection of several exogenous amino acids such as kainic acid (Coyle and Schwarcz, 1976). Quinolinic acid, an endogenous metabolite of tryptophan, produces similar changes, thus suggesting that it could play a role in the pathogenesis of Huntington's disease (Björklund et al., 1986). The marked increase of the anabolic enzyme for quinolinic acid in both experimental excitotoxic lesions and in Huntington's disease suggests that astrocytes are primarily involved in quinoline production, since they are the main cell population in these conditions and that they could possibly be responsible for neuronal damage.

HISTOPATHOLOGY

Protoplasmic Gliosis

Protoplasmic gliosis is not associated with the formation of glial fibers and is the characteristic finding of hepatic encephalopathy; this is covered in Chapter 63. Two other lesions traditionally considered as examples of protoplasmic gliosis are the *Gliarasen* (Figure 56-2) and neuronal satellitosis (Figure 56-3). Both lesions characteristically occur in the cerebral cortex and in-

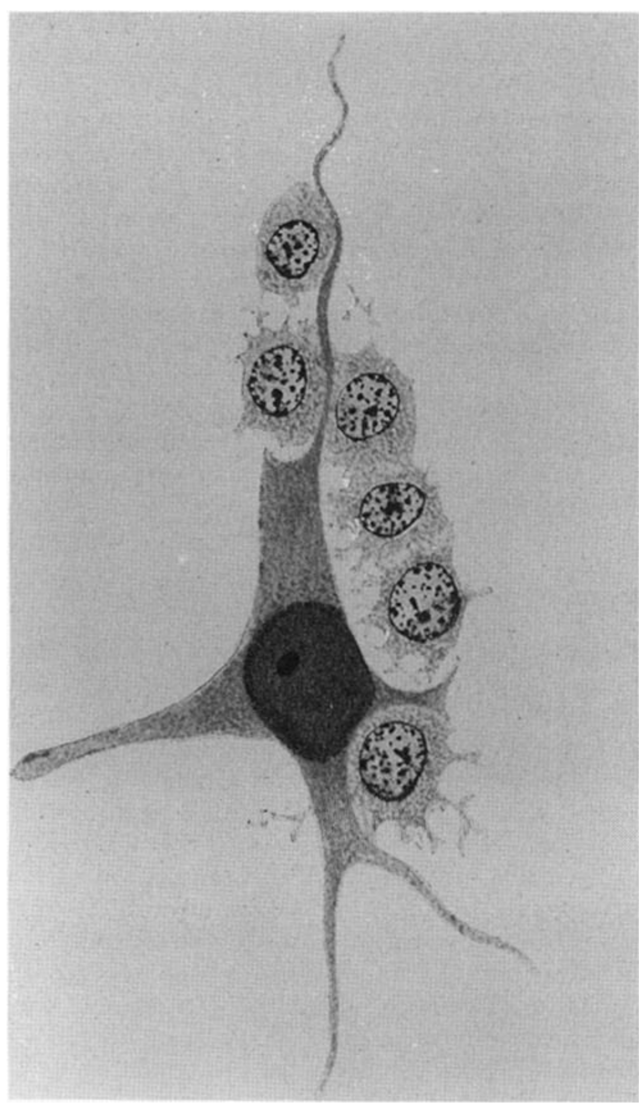


FIG. 56-3. Neuronal satellitosis. [From Spielmeyer (1922).] See Chapter 59 in this volume for the astrocyte reaction to neuronal damage *not* resulting in cell necrosis.

dicate neuronal damage. It should be noted that the absence of glial fibers was assessed by the method of Weigert and its modifications, and it is thus possible that astrocytes forming the lesions are, in fact, GFAP-positive, since GFAP immunohistochemistry is a more sensitive procedure. Conversely, the absence of GFAP in the astrocytes showing the characteristic changes of hepatic encephalopathy (Alzheimer's type II astrocytes) has been confirmed by immunohistochemistry (see Chapter 63).

Cell Division

The *Gliarasen,* pairs or quartets of astrocyte nuclei surrounded by thick protoplasmic processes (Figure 56-2), originally suggested that reactive astrocytes are capable of true hyperplasia, that is, cell division. Since mitotic figures are difficult to see in autopsy material, probably because most of them reach completion in the time interval between death and fixation, it was mistakenly believed that astrocyte division was amitotic.

Definite proof that division of reactive astrocytes actually occurs was difficult to obtain because [^{3}H]thymidine autoradiographic products were bleached by the method traditionally employed to stain astrocyte perikarya, that is, Ramón y Cajal's gold sublimate. Thus, it was difficult to ascertain the identity of the cells incorporating [^{3}H]thymidine in a brain wound, whether macrophages, fibroblasts, or astrocytes (Lindsay, 1986). Ultrastructural studies of pulse-labeled cells also did not provide an unequivocal answer, since macrophages, like astrocytes, may contain intermediate filaments. The question was conclusively answered in the affirmative by combining GFAP immunohistochemistry with tritiated thymidine autoradiography (Latov et al., 1979; Miyake et al., 1988; Janeczko, 1989). Incorporation of tritiated thymidine by reactive astrocytes is an early phenomenon, occurring as soon as 2 hours after injury and thus preceding GFAP expression (Janeczko, 1989). It should be noted, however, that upregulation of GFAP and fibrillogenesis are the main factors in the formation of the glial scar. Astrocyte proliferation is a relatively limited phenomenon mainly confined at the site of injury (Miyake et al., 1992).

Astrocyte division mainly occurs in brain injury associated with disruption of the blood-brain barrier. The number of astrocytes remained practically constant in rat optic nerve undergoing Wallerian degeneration (Skoff and Vaughn, 1971). Also, [^{3}H]thymidine labeling of GFAP-positive cells was not observed in spinal cord following rhizotomy, notwithstanding the presence of severe gliosis along the pathway of the degenerating dorsal root axons (Murray et al., 1990).

Isomorphic and Anisomorphic Fibrous Gliosis

Glial fibers are condensed but basically maintain their normal orientation (isomorphic gliosis, Figures 56-4 and 56-5); alternatively, glial architectonics is severely disrupted (anisomorphic gliosis, Figure 56-6). Isomorphic gliosis is typically observed in cases of selective damage to neurons and their processes. Conversely, severe tissue damage usually, but not invariably, associated with disruption of the blood-brain barrier, results in anisomorphic gliosis. Isomor-

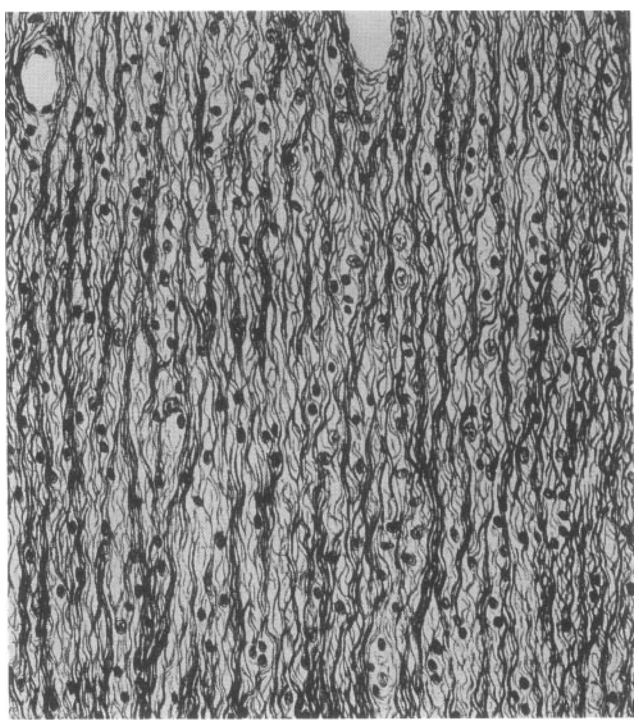


FIG. 56-4. Isomorphic gliosis of a degenerated spinal cord fiber tract. Weigert's staining for glial fibers. Note that fibrous astrocytes maintain their normal longitudinal orientation in the degenerated tract (compare with Figure 56-6). [From Spielmeyer (1922), with permission.]

phic and anisomorphic gliosis are easily distinguished in regions of the brain where glial fibers are regularly oriented, e.g., in myelinated tracts (Figure 56-4) and in the cerebellar cortex (Figure 56-5). Conversely, the distinction is not easy where glial fibers normally run an irregular course (Figure 56-7).

In isomorphic gliosis, the morphological findings do not provide incontrovertible evidence as to a real increase of glial fibers, since they could suggest a simple condensation of the glial framework as a result of the loss of nerve. The study of Kost-Mikucki and Oblinger (1991) is thus important in this respect because it shows significant increase of GFAP mRNA in pyramidal tracts undergoing Wallerian degeneration, a condition typically associated with isomorphic gliosis.

An interesting question is whether the dense glial scar characteristic of anisomorphic gliosis is the product of astrocytes originally located at the site of injury. A possibility to be considered is that astrocytes migrating from peripheral areas also participate in the process. In fact, the studies of Janeczko (1989) seem to point in this direction in that [^{3}H]thymidine labeled astrocytes, at first scattered over a relatively wide area, later became concentrated around the lesion. Astrocytes derived from primary cultures or from fetal central nervous system tissue are capable of extensive migration when transplanted to mature brain (see Zhou et al., 1990, for references), up to 5 cm in the spinal cord according to one report (Goldberg and Bernstein, 1988). Experiments with peripheral ganglia implants suggest that reactive astrocytes are also capable of migration (Rosenstein and Brightman, 1981; Dahl et al., 1991). Astrocytes were able to invade the grafted ganglia as evidenced by a positive reaction to GFAP antibodies only reacting with the "central" form of GFAP (Jessen et al., 1984) (Figure 56-8). Another point of interest was that reactive astrocytes did not appear to affect sensory neurons in DRG implants (Figure 56-9).

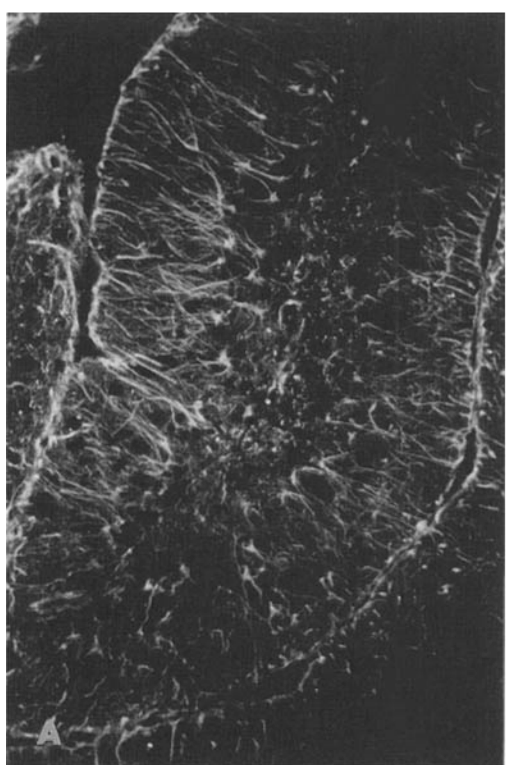
Gemistocytic Astrocytes

Large astrocytes with homogeneous cytoplasm (Figure 56-10) were called *Gemästate* (fattened) by Nissl and they are often referred to as gemistocytic astrocytes in the English literature. These cells are particularly numerous in regions undergoing severe fibrous gliosis and they were thus traditionally considered as the fiber-forming cells. Brain cytoskeletal proteins, including GFAP, are synthesized by free polysomes (Strocchi et al., 1982). Gemistocytic astrocytes share common features with neurons undergoing central chromatolysis, a basic reaction to axonal transection. Nissl granules consisting of parallel cisterns of rough endoplasmic reticulum are replaced by free ribosomes under this condition.

Formation of Accessory Glial Limiting Membranes

Astrocytes form the boundary between central nervous system and non-central nervous system tissues in both normal and pathological conditions. In normal brain, a continuous lining of astrocyte processes covered by a basal lamina is interposed between brain and meninges (external glial membrane) and between brain and blood vessels (perivascular glial membrane).

As to the formation of glial membranes in pathological conditions, a brain infarct can be considered as an example. Starting a few days after ischemic necrosis, reactive astrocytes surround the infarct with a thick mesh of fibrils, thus demarcating the necrotic tissue from viable brain and probably preventing the spread of edema. After a period of time that may vary from weeks to months depending on the size of the infarct, the glial scar covered by a basal lamina forms the new surface of the brain. The



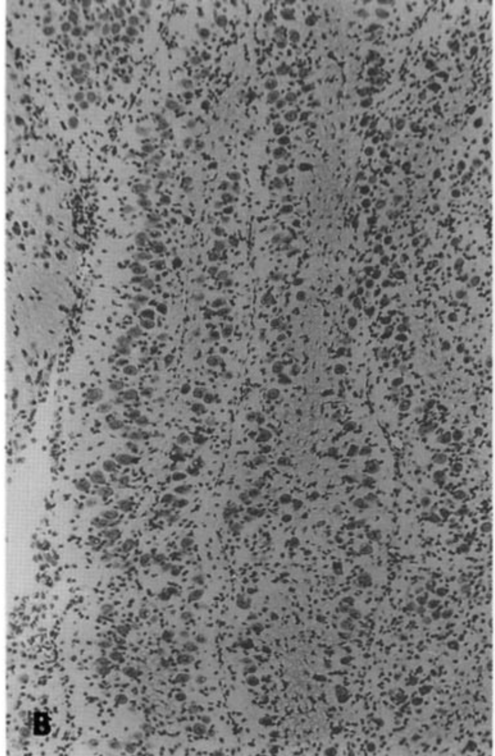


FIG. 56-5. (A) Isomorphic gliosis of cerebellum in 1-month-old homozygous weaver, a mouse mutation leading to the loss of the granule cell layer. (B) Note that the glial fibers maintain the general orientation of Bergmann glia in the atrophic cerebellum. Figure A: immunofluorescence with GFAP antiserum; B: toluidine blue stain. [From Bignami and Dahl (1974c), with permission.]

cyst resulting from the absorption by bloodborne macrophages of the necrotic tissue is now part of the subarachnoid space (Figure 56-11). If the infarct is deep in the brain, in cerebral white matter or in basal ganglia, the cyst is part of the perivascular space (Figure 56-12). Indirect evidence suggests that astrocytes may participate in the formation of the basal lamina. Astrocytes in primary culture synthesize variant forms of two basal membrane proteins, that is, laminin and fibronectin (Liesi et al., 1983; Price and Hynes, 1985; Liesi and Risteli, 1989). Astrocytes in culture also produce chondroitin sulfate proteoglycan (Gallo and Bertolotto, 1990) but it is still not known whether this proteoglycan is the heparan sulfate proteoglycan of basal laminae. According to a recent paper, post-translational modification of this proteoglycan may include substitution with chondroitin sulfate side chains (Murdoch et al., 1992).

Glycogen

In normal brain, glycogen is predominantly stored in astrocytes. Binding of norepinephrine and vasoactive intestinal peptide (VIP) to their receptors stimulates glycogenolysis, and this is also true for several substances released by neurons during depolarization such as adenosine and potassium. This suggests that astrocytes may help neurons with their energy requirements during periods of activity (Magistretti et al., 1986; Chapter 52, this volume).

Glycogen accumulation after an initial decrease is a distinctive feature of astrocytes responding to injury (for review see Haymaker et al., 1970). The process leading to glycogen accumulation is still unknown. An induction of glycogen synthesis resulting in a marked increase of glycogen concentration has been observed after VIP- and norepinephrine-induced glycogenolysis in primary astrocyte cultures (Sorg and Magistretti, 1992).

UPREGULATION OF GFAP EXPRESSION: A SENSITIVE INDICATOR OF BRAIN INJURY

GFAP is the subunit of glial-specific intermediate filaments and it is thus not surprising that GFAP expression is enhanced in reactive astrocytes. As noted above, accumulation of glial filaments is the characteristic finding of fibrous gliosis.

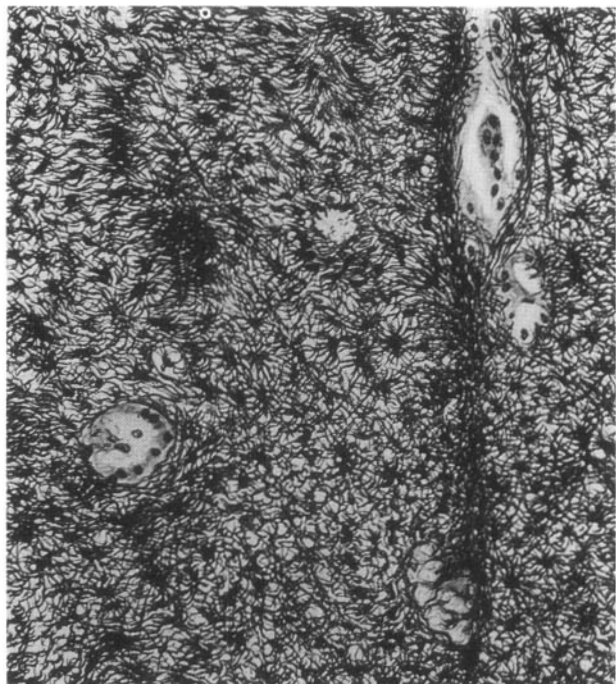


FIG. 56-6. Anisomorphic gliosis in an old multiple sclerosis plaque. Weigert's stain for glial fibers. [From Spielmeyer (1922), with permission.] Astrocytes from a dense mesh of fibers with no discernible pattern (compare with Figure 56-4).

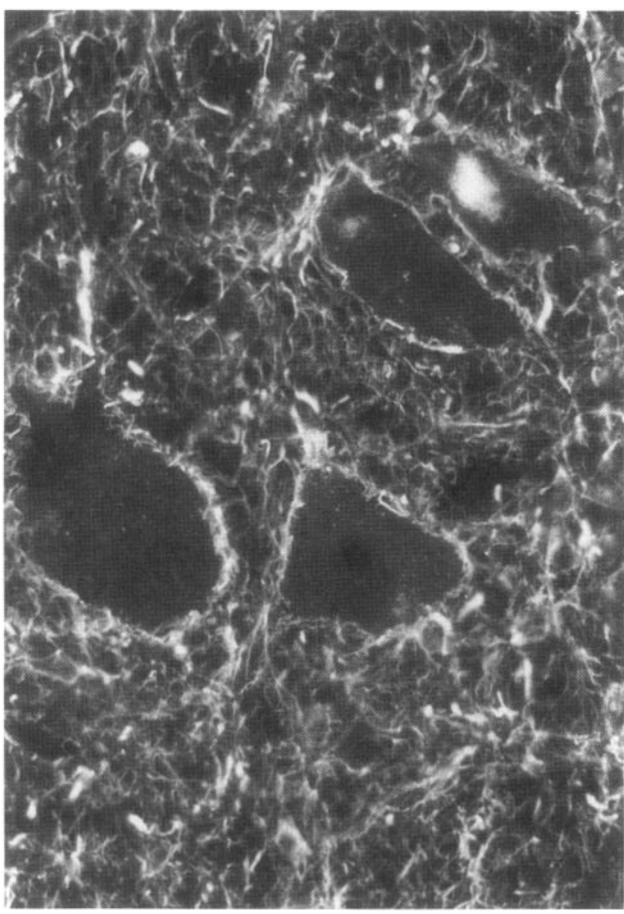


FIG. 56-7. Normal dog spinal cord gray matter stained with GFAP antiserum. Glial fibers form a delicate mesh with no recognizable orientation. The distinction between isomorphic and anisomorphic gliosis is practically impossible in such a location.

The factors that induce GFAP expression in development are probably different from those that operate in reactive astrocytes (Sarthy et al., 1991). Protoplasmic astrocytes in rat cerebral isocortex are GFAP-negative, but they convert into cells that stain brightly with GFAP antibodies 2 days after injury (Figure 56-13), mRNA for GFAP becoming maximal at 4 to 5 days (Cancilla et al., 1992). The same is true for Müller glia in the rat retina (Figure 56-14). The question may be raised as to whether GFAP is produced *ex novo* under these conditions or whether its production is upregulated, basal levels being too low for detection. We note that in the case of Müller glia, the negative immunohistological findings have been confirmed by *in situ* hybridization studies of GFAP mRNA expression, and it has also been shown that the GFAP gene is transcriptionally activated in Müller cells of mice with retinal degeneration (Sarthy et al., 1991). In mice as in rat, Müller glia convert into GFAP-positive cells in retinal detachment and degeneration (Dräger and Edwards, 1983; Shaw and Weber, 1983; Eisenfeld et al., 1984; Erickson et al., 1987). Interestingly, Müller cells in chick retina, which are normally GFAP-positive do not accumulate GFAP in response to degeneration (Semple-Rowland, 1991).

Since GFAP is the subunit of the filaments forming the glial fiber, increased immunohistological staining for GFAP is the equivalent of fibrous gliosis. However, GFAP immunohistology is much more sensitive than the traditional staining procedures for glial fibers, and it has been used to study astrocyte reactions to injury that would have been difficult to detect otherwise. In fact, this astrocyte response is often easier to demonstrate than the underlying brain damage. An important point raised by these studies is that gliosis is not necessarily a permanent phenomenon, as originally believed when it could only be demonstrated in areas of severe tissue injury. Transient gliosis, that is, increased GFAP immunostaining followed by return to normal levels, has been observed after excitotoxin lesions (Björklund et al., 1986), deafferentation (Gage et al., 1988; Anders and Johnson, 1990), transganglionic degeneration (Hajós et al., 1990), spreading depression (Kraig et al., 1991), chronic hypoxia (Zimmer et al., 1991)

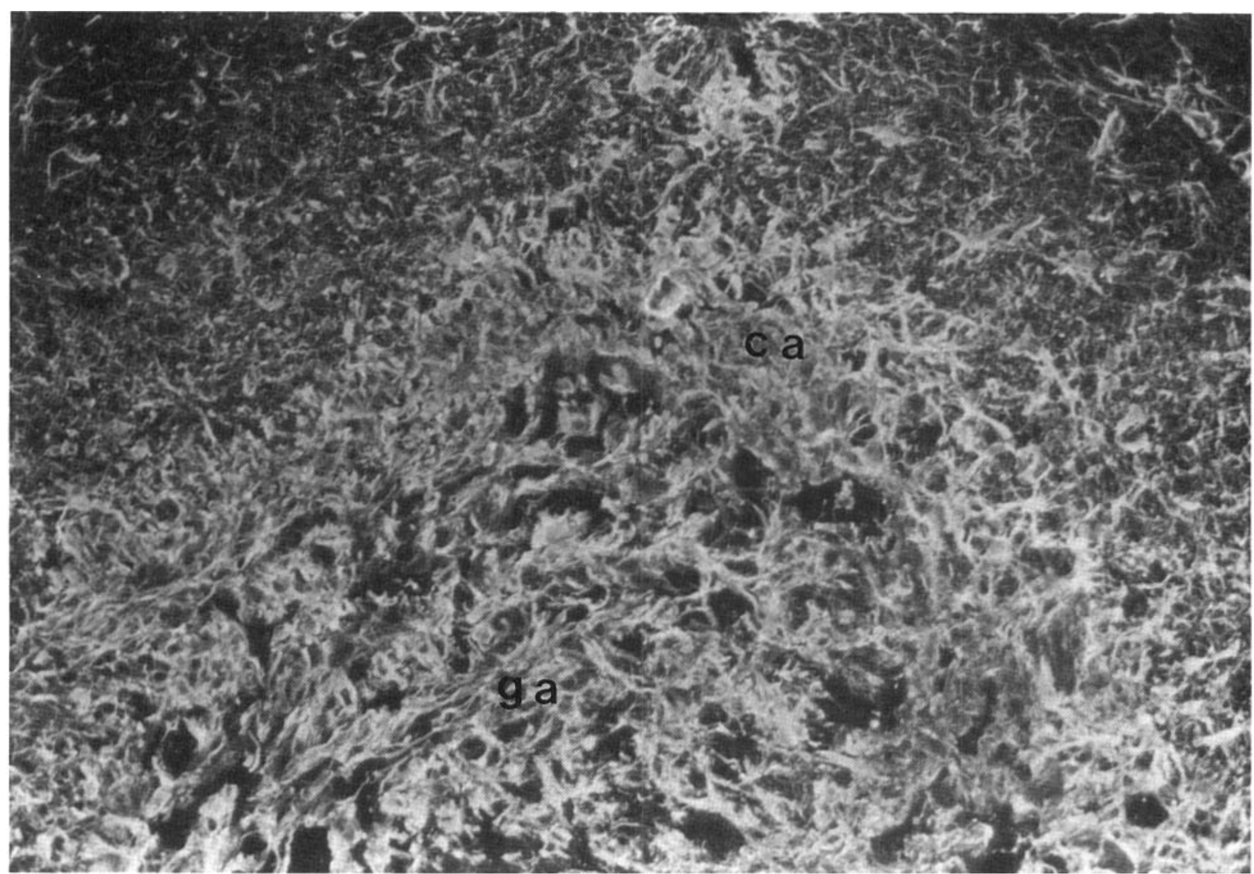


FIG. 56-8. Dorsal root ganglion grafted in rat cerebral hemisphere 1 month after operation and stained with a GFAP monoclonal antibody not reacting with the peripheral form of GFAP. The ganglion (*ga*) is surrounded by a dense glial capsule (*ca*). [From Dahl et al. (1991), with permission.]

and cortical devascularization except for the area surrounding the devascularized area (Herrera and Cuello, 1992).

Axonal Reaction

Gliosis that follows the reaction of the nerve cell to a lesion of its axons is not covered here because it is better discussed in association with the microglial changes (see Chapter 59). We only note that when the axonal reaction results in cell death, severe gliosis may occur which is readily detectable by traditional staining methods for glial fibers, for example, the severe gliosis of the thalamic nuclei relaying to the frontal cortex in patients with prefrontal leucotomy.

Terminal Degeneration (Deafferentation)

Electron microscopic studies of the lateral geniculate body after eye removal in the squirrel monkey showed that astrocyte processes were greatly enlarged and contained many filaments. Furthermore, they were seen to enwrap dense degenerated terminals (Wong-Riley, 1971). With GFAP antibodies, an astrocyte reaction was first detected one day after deafferentation in rat superior colliculus. At 21 days, a dense network of GFAP-positive fibers had become apparent (Schmidt-Kastner et al., 1993).

The astrocyte response to denervation has been particularly well studied in the dentate gyrus of the hippocampus, and this is probably because of the remarkable sprouting and heterotypical innervation that results from the lesion (Rose et al., 1976; Gage et al., 1988; Tanaka et al., 1991). The dramatic increase of GFAP immunoreactivity, which in the case of perforant path lesions selectively involves the outer molecular layer of the dentate gyrus, suggests that reactive astrocytes secrete neurotrophic factors as a necessary requirement for the sprouting response (Gage et al., 1988). Surprisingly, the expression of GFAP mRNA after lesions of the entorhinal cortex was not confined to the denervated part of the dentate gyrus but also involved areas that do not

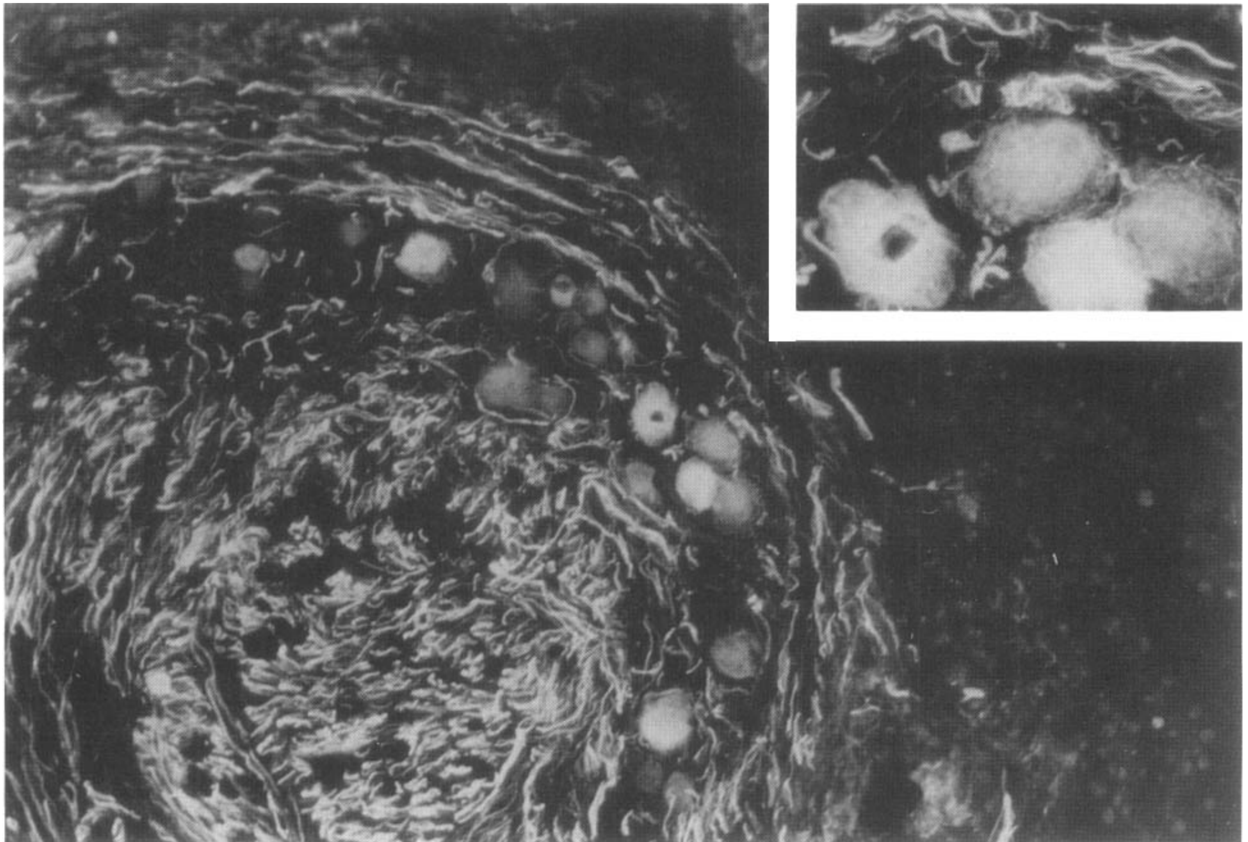


FIG. 56-9. Dorsal root ganglion (*DRG*) grafted in rat cerebral hemisphere 1 month after operation and stained by indirect immunofluorescence with a neurofilament monoclonal antibody. Note the normal appearance of DRG neurons. **Inset:** DRG neurons at higher magnification. [From Dahl et al. (1991), with permission.]

receive projections from the entorhinal cortex (Steward et al., 1990). Upregulation of GFAP mRNA levels was not dependent on induction of immediate early genes (Smith and Steward, 1992).

A vigorous astrocyte response to denervation was also observed in rat olfactory bulb (Anders and Johnson, 1990). The olfactory nerve is capable of functional regeneration after axotomy, thus suggesting that, as in the dentate gyrus of the hippocampus, reactive astrocytes are compatible with axonal growth.

Transganglional Degeneration

Sciatic nerve section or compression in the rat is followed by a marked increase of GFAP immunoreactivity occurring as early as 3 days after the lesion in the segmentally related ipsilateral dorsal horn (Hajós et al., 1990; Murray, 1990; Garrison et al., 1991). According to Hajós et al. (1990), the gliosis is due to degenerative changes of the central primary afferent terminals resulting from blockade of retrograde nerve growth factor transport in the injured nerve. In agreement with this hypothesis, gliosis markedly decreased as soon as the regenerating peripheral axons had reached their target.

"Functional" Damage

Increased GFAP immunoreactivity has been also reported in the apparent absence of structural damage, specifically in the hypoglossal nucleus after electrical stimulation of the hypoglossal nerve (Hall et al., 1989), in the hippocampus after repeated sessions of electroconvulsive shock (Orzi et al., 1990), in spreading depression induced by the application of potassium chloride to the cerebral cortex (Kraig et al., 1991) and in kindling, a permanent seizure disorder resulting from the daily application of electrical stimuli to susceptible parts of the brain (Khurgel et al., 1992). Electrically induced seizures in rat hippocampus also resulted in a rapid and dramatic increase in mRNA for GFAP (Steward et al., 1991).

Still little is known concerning the disruption of functional glial-neuronal interactions that may occur when astrocytes respond to injury. An example

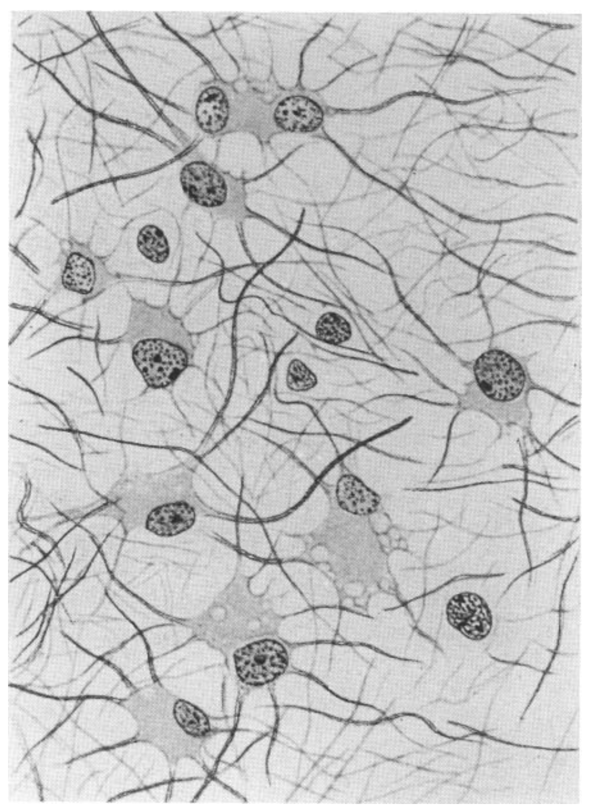


FIG. 56-10. Gemistocytic astrocytes in cerebral cortex. Weigert's stain for glial fibers. [From Spielmeyer (1922).]

would be the glutamate uptake system in reactive astrocytes. If uptake is enhanced, the neurons would get extra protection against the toxic effect of the excitatory neurotransmitter (see Chapter 48). Alternatively, the uptake system may be impaired, considering that the astrocyte machinery is switched to other tasks such as fibrillogenesis.

VIMENTIN IN REACTIVE ASTROCYTES

The protein composition of rat optic nerve in wallerian degeneration first suggested that vimentin accumulates in reactive astrocytes (Dahl et al., 1981a). The two main proteins in the gliosed nerves were GFAP and a 57 kD protein, which was identified as vimentin, the fibroblast-type intermediate filament protein (Figure 56-15). Furthermore, vimentin immunoreactivity was markedly increased compared to normal optic nerve (Figure 56-16).

Immature glia first express vimentin before they switch to astrocyte-specific GFAP (Dahl et al., 1981b), and the question may be asked whether the same is true for reactive astrocytes. This, in fact, does not appear to be the case, since in stabbed cerebral cortex vimentin is only expressed by astrocytes located in the vicinity of the mesenchymal scar while

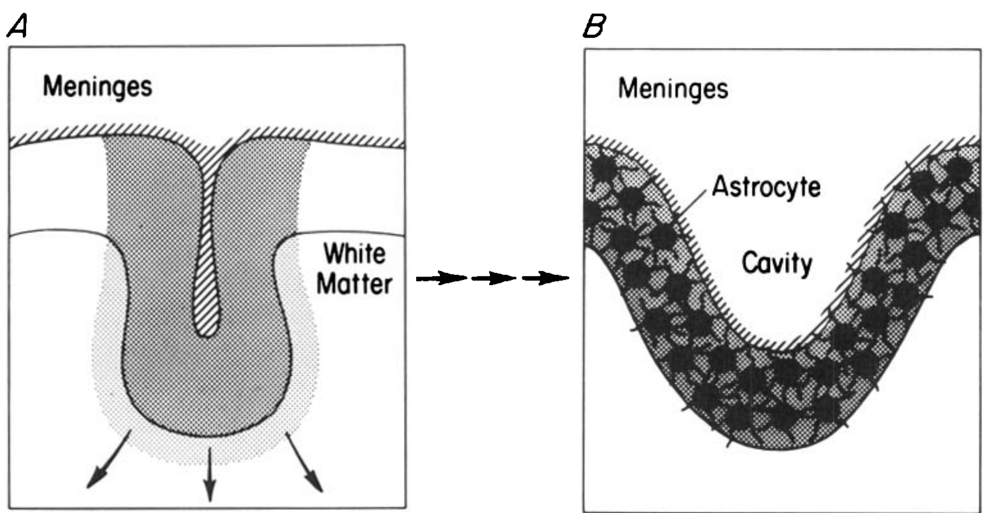


FIG. 56-11. **(A)** Cerebral infarct involving cortex and white matter. *Arrows* point to the spread of edema into the underlying white matter. **(B)** Final stage of the corticosubcortical infarct shown in Figure A. An astrocyte scar surrounds the cavity resulting from absorption of necrotic tissue by bloodborne macrophages. Note that the cavity is now part of the subarachnoid space and, as in normal cortex, the astrocytic scar is covered by a basal lamina (*dashed line*). [From Bignami (1991), with permission.]

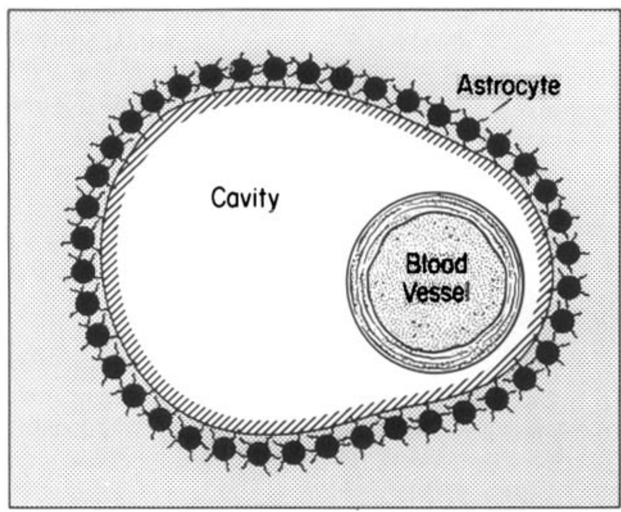
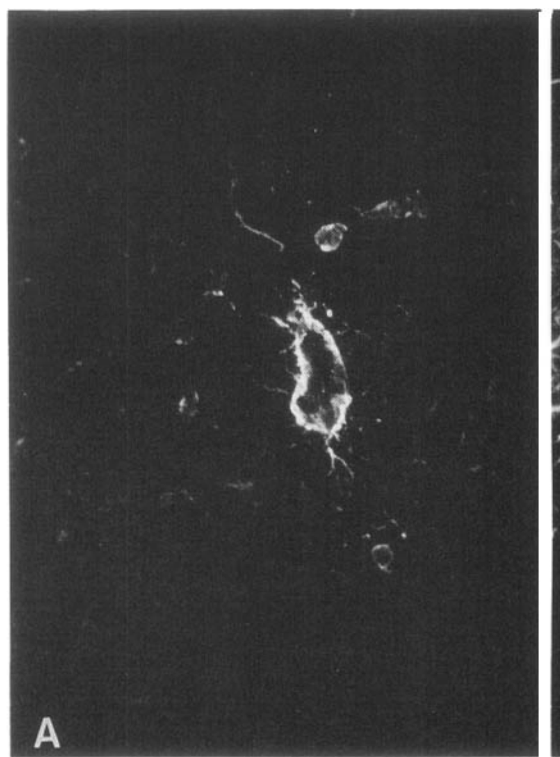


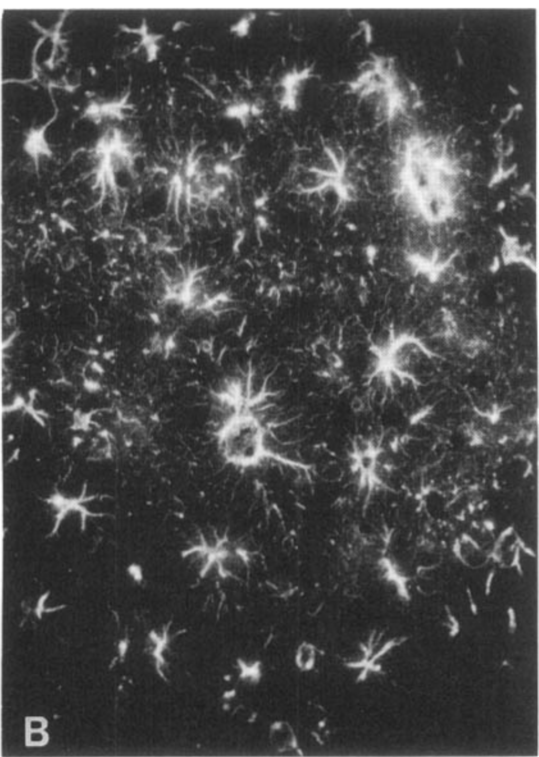
FIG. 56-12. If the infarct is deep inside the brain (e.g., in white matter or basal ganglia) the cavity resulting from reabsorption of the necrotic tissue is now part of the perivascular space. Such small cysts are often referred to as lacunar infarcts. The *dashed line* surrounding the cavity is the basal lamina.

the astrocyte reaction as assessed by GFAP immunoreactivity extends far beyond (Pixley and deVellis, 1984; Schiffer et al., 1986; Mansour et al., 1990; Calvo et al., 1991). Conversely, white matter astrocytes which normally contain GFAP and, to a lesser extent, vimentin, accumulate both GFAP and vimentin (Mansour et al., 1990; Oblinger and Singh, 1993).

EXTRACELLULAR MATRIX

Compared to other tissues, surprisingly little is known about the composition of extracellular matrix in the brain. Perhaps the most important factor for the lack of research on this topic is the electron microscopic appearance of "well fixed" brain tissue. In these microphotographs brain extracellular space is markedly reduced from its physiological size, accounting for approximately 17 to 20% of the brain





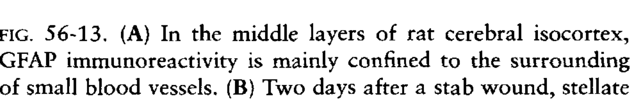


FIG. 56-13. (**A**) In the middle layers of rat cerebral isocortex, GFAP immunoreactivity is mainly confined to the surrounding of small blood vessels. (**B**) Two days after a stab wound, stellate astrocytes are readily apparent. [From Bignami and Dahl (1974b), with permission.]

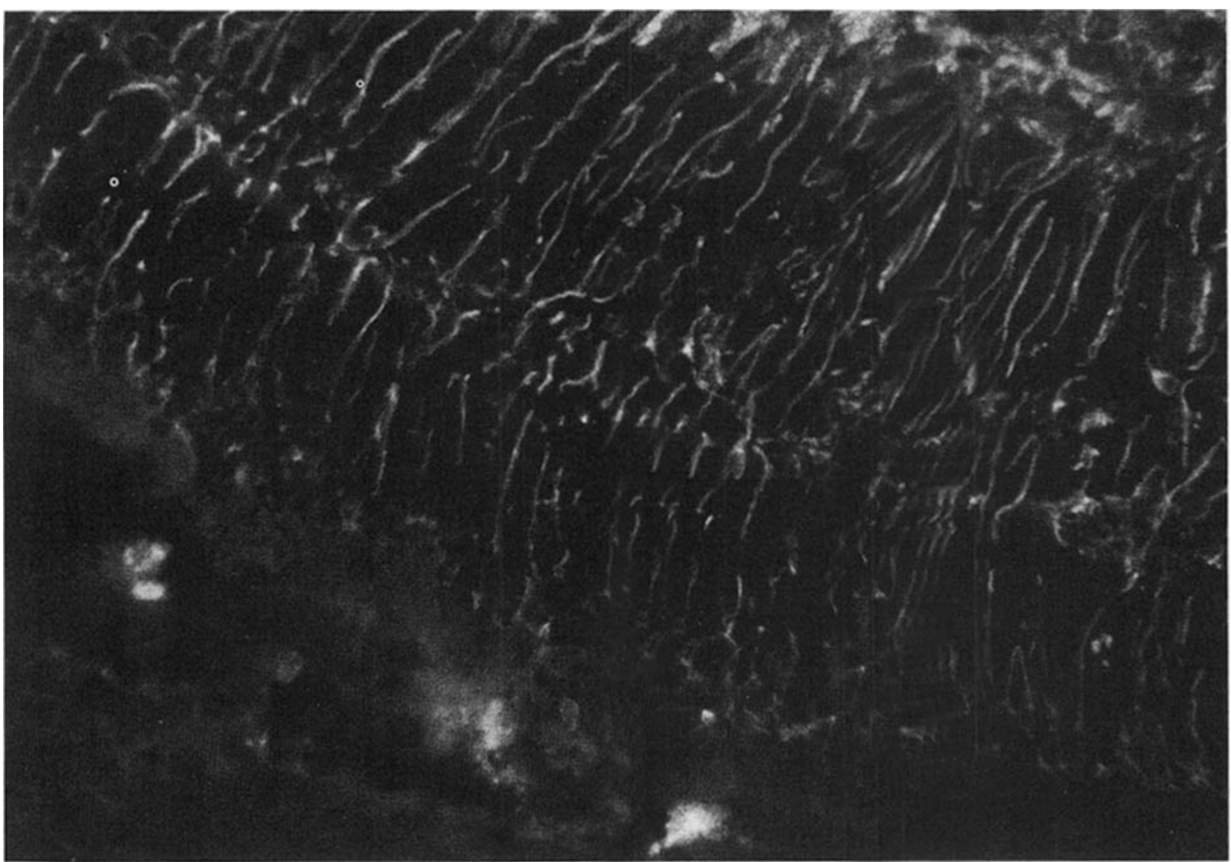


FIG. 56-14. Immunofluorescent staining with GFAP antiserum of the radial fibers of Müller in rat retina following optic nerve crush. [From Bignami and Dahl (1979), with permission.] In normal rat retina, GFAP immunoreactivity is confined to astrocytes in the ganglion cell and nerve fiber layers (see Chapter 43).

volume, to about 5%. Furthermore, this space appears empty. In accordance with light microscopic observations, it does not contain structures found extracellularly in other tissues, such as fibrils and basal membranes. Also, it is devoid of the electron dense material that one would expect if it contained proteins. It has now been shown that the apparent emptiness of brain interstitium is due to the fact that in brain, as in cartilage, hyaluronic acid (HA) plays a structural role in the formation of the extracellular matrix (for review see Bignami et al., 1993). The solubility of HA in aqueous fixatives is a well-known phenomenon. Furthermore, a 60 kD HA-binding protein is released intact from brain homogenates by hyaluronidase digestion, thus suggesting that it will also disappear from the sections if HA is dissolved (Asher et al., 1991). The protein was called glial HA-binding protein (GHAP) and this in view of the fact that its distribution is similar to that of GFAP at the light microscopic level. Amino acid sequence analysis indicates that GHAP derives from versican, a large chondroitin sulfate proteoglycan of precartilaginous mesenchyma either by limited proteolysis or alternative splicing. GHAP is mainly confined to white matter, while versican is found in both white matter and gray matter. In gray matter it forms characteristic perineuronal coats (the Golgi pericellular net) together with aggrecan, the chondrotin sulfate proteoglycan of mature cartilage.

HA and GHAP persist for long periods of time in white matter tracts and optic nerves undergoing Wallerian degeneration and isomorphic gliosis (Bignami et al., 1989) (Figure 56-17). The more pronounced staining of gliosed compared to normal optic nerve shown in Figure 56-17 could be due to the condensation of the extracellular matrix resulting from the loss of myelinated axons rather than increased synthesis. Conversely, we have not been able to detect GHAP and hyaluronic acid in dense anisomorphic white matter glial scars resulting from penetrating wounds and multiple sclerosis suggesting that glial processes in these scars are closely packed with little intercalated extracellular matrix (Dahl et al., 1989; Mansour et al., 1990, unpublished observations). If in cortical scars such loss of extracellular matrix also includes proteoglycans, it is tempting to

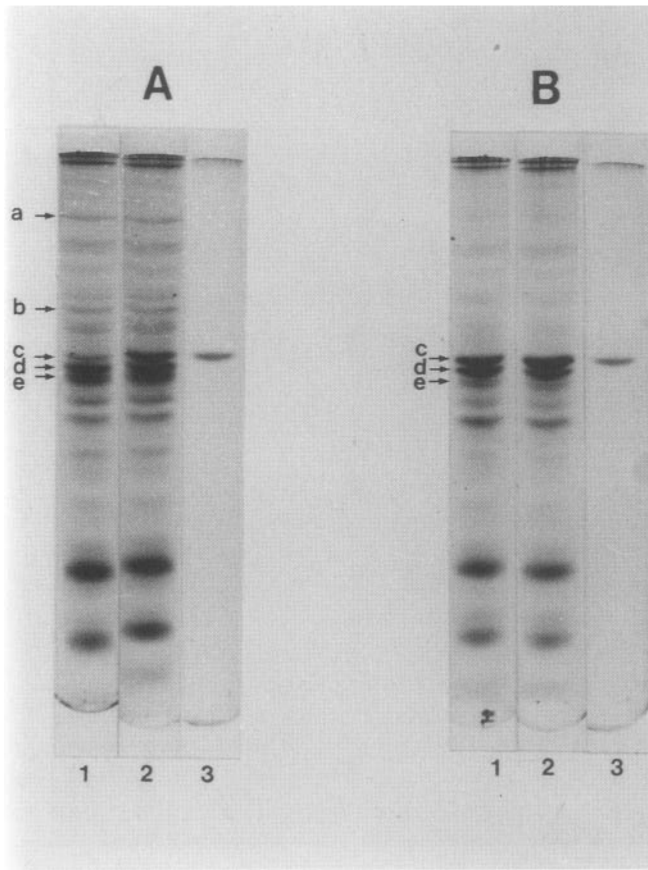


FIG. 56-15. Identification of vimentin in rat optic nerves, normal and degenerated, by SDS-PAGE. (A) Gel 1, normal optic nerve. Identified bands: *a*, 150,000 kD neurofilament protein; *b*, 70 kD-neurofilament protein; *c*, vimentin; *d*, GFAP; *e*, tubulinin. Gel 2, mixture of gel 1 with rat vimentin. Gel 3, rat vimentin. (B) Gel 1, optic nerve 3 weeks after enucleation. Identified bands as in Figure A. Gel 2, mixture of Gel 1 with vimentin. Gel 3, vimentin. Note in the degenerated nerves the disappearance of neurofilament proteins and the marked increase in vimentin. [From Dahl et al. (1981a), with permission.]

speculate that the phenomenon may contribute to the decrease in interstitial calcium that precedes seizures in epileptogenic foci and that would increase neuronal excitability (Dietzel and Heinemann, 1986). It is reasonable to assume that the negatively charged proteoglycans play a role in retaining divalent calcium (Ca^{2+}) in the neuronal microenvironment.

GLIOSIS IN IMMATURE BRAIN

The question of gliosis resulting from injury to immature brain, for example, before the onset of myelination, remains a matter for further investigation. In accordance with Berry et al. (1983), we found relatively little gliosis, as evidenced by GFAP immunohistochemistry, in rats stabbed at birth and sacrificed 2 days later (Bignami and Dahl, 1976). However, severe and diffuse gliosis was observed after 13 days or later, in accordance with Janeczko's (1988) finding of an extensive astrocyte proliferative response to injury in neonatal rat brain. Compared to adult rats, the distinctive features of stabbed newborn rats sacrificed 1 month after operation were the presence of white matter cavitation and the absence of a mesenchymal scar at the site of the stab. Similar results have been reported by Berry et al. (1983). Conversely, severe reactive gliosis as shown by GFAP immunoreactivity has been reported in various types of pre- and perinatal damage in the human brain by Roessmann and Gambetti (1986).

Differences have also been reported between adult and newborn rats in their astroglial response to spinal cord injury and dorsal root transection, gliosis in the spinal cord being much less intense in the newborn animals (Barrett et al., 1984; Murray et al., 1990).

While the extent and time course of gliosis following perinatal brain wounds remain a controversial issue, no differences have been shown between neonatal and mature astroglia in their response to Wallerian degeneration of rat optic nerve and hamster corticospinal tract, that is, isomorphic gliosis (Fulcrand and Privat, 1977; Trimmer and Wunderlich, 1990; Oblinger and Singh, 1993). In adult and newborn animals the end result was the formation of a dense glial scar occurring at a much earlier date in newborn animals. This probably in view of the fact that in Wallerian degeneration of adult central nervous system tracts the removal of myelin debris is a lengthy process (Bignami and Ralston, 1969).

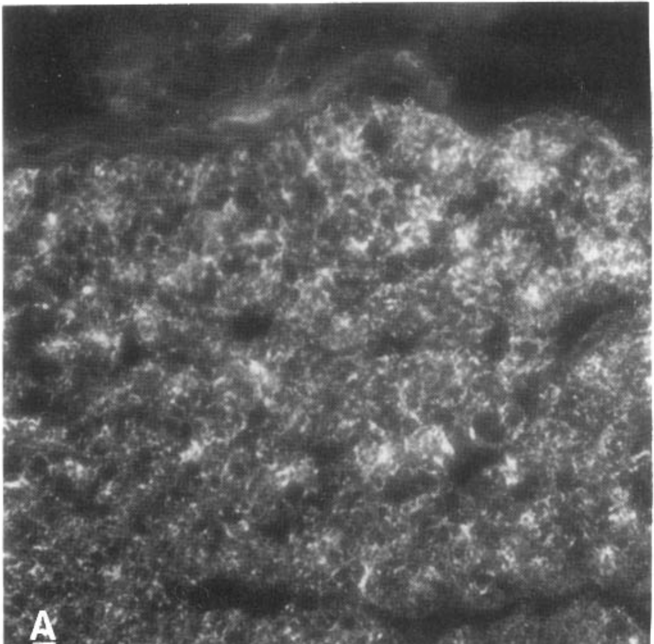
GLIOSIS IN SUBMAMMALIAN VERTEBRATES

Little is known about the astrocyte response to injury in submammalian vertebrates although perhaps it is an important topic, especially in view of the inherent capacity for regeneration of the central nervous system in fish and amphibia and of the putative role of gliosis in preventing regeneration of mammalian central nervous system tracts (Reier et al., 1983).

We have studied gliosis in goldfish spinal cord and we found that the regenerated spinal cord at the site of transection was almost completely devoid of GFAP immunoreactive material (Bignami et al., 1974). Conversely, gliosis was observed in the proximal and distal stumps of the transected spinal cord when their reunion was prevented.

Similar findings have been reported in goldfish op-

FIG. 56-16. (**A**) Staining of leptomeninges and glial septa in a transverse section of normal rat optic nerve. Immunofluorescence with vimentin antisera. (**B**) Intense staining of gliosed rat optic nerve 1 month after enucleation. Immunofluorescence with vimentin antisera. [From Dahl et al. (1981a), with permission.]



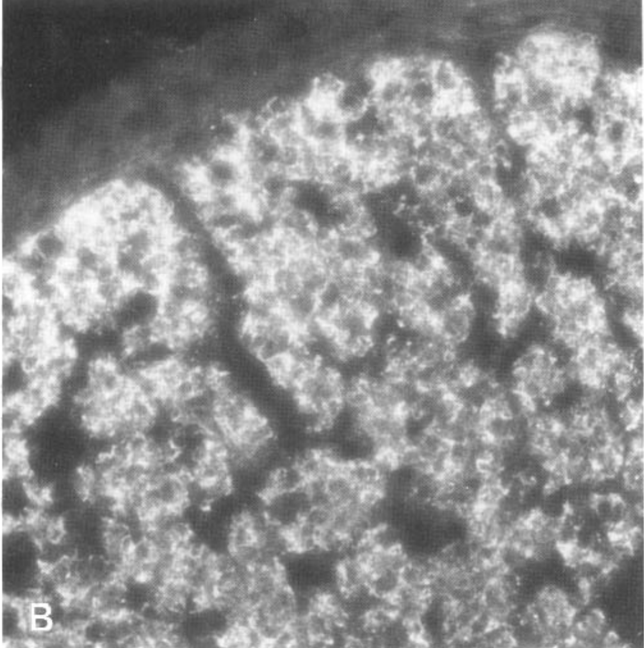


FIG. 56-17. Condensation of the extracellular matrix in rat optic nerve undergoing wallerian degeneration 55 days after crush. (**A**) Normal optic nerve is stained for hyaluronic acid (Bignami and Asher, 1992). (**B**) Degenerated optic nerve is stained with antibodies to the glial hyaluronate-binding protein.

tic nerve by Stafford et al. (1990). An interesting observation was that optic nerve astrocytes, which normally do not stain with GFAP antibodies (Dahl and Bignami, 1973b) and express keratin (Giordano et al., 1990) had become GFAP-positive after transection. More recently, it was shown that the GFAP-negative regenerated optic nerve filling the gap between proximal and distal stumps was populated by Schwann-like cells and thus bore a strong resemblance to peripheral nerve (Nona et al., 1992).

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57 Oligodendrocyte inhibition of nerve fiber growth and regeneration in the mammalian central nervous system

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In contrast to most organs of the body, which retain a life-long potential for renewal and plasticity, cell division does not occur in the adult central nervous system, and outgrowth of nerve fibers is extremely restricted. Many of the long-distance connections and fiber tracts are formed prenatally, and the postnatal differentiation of the nervous system includes a progressive restriction of fiber growth. In the adult, plastic changes in dendritic trees and axonal arborizations do occur and may in fact be much more common than previously assumed (Purves et al., 1986; Benowitz et al., 1988; Rossi et al., 1991, Woolf et al., 1992). However, the distances of growth are usually restricted to a few hundred microns, and rarely exceed 1 mm. Reactions to lesions of central fiber tracts in higher vertebrates parallel these developmental events: whereas fiber tracts can regenerate in very immature animals, this process is strongly restricted in later development and absent in most central nervous system fiber systems in the adult (Kalil and Reh, 1982; Bernstein and Stelzner, 1983; Shimizu et al., 1990; Hasan et al., 1991, Treherne et al., 1992). Although regenerative sprouting can occur, the length of these sprouts remains limited, and elongation beyond 1 mm does not occur.

In the search for an explanation of the limited fiber growth and regeneration potential of the adult central nervous system, the first hypothesis to be formulated and experimentally tested was that of an intrinsic inability of adult neurons to grow (for review see Ramón y Cajal, 1928). Since peripheral nerves (in particular following crush lesions, which are more conducive than transections) were well known for their successful growth of sensory and motor fibers over long distances, peripheral nerve grafts were placed into various areas of the central nervous system. These experiments allowed exposure of central neurons to a peripheral, regeneration-permissive tissue environment. The first successful experiments were published by F. Tello in 1911, and showed that nerve fibers from the rabbit neocortex invaded implants of peripheral nerves. Tello and later Ramón y Cajal (1928), therefore drew the conclusion that adult central nervous system neurons can regenerate their axons, but that the *tissue environment* is of crucial importance. They hypothesized that denervated Schwann cells produced trophic factors which attracted and sustained the regenerating axons. Interestingly, their results were reinterpreted by Le Gros Clark (1943) in light of the newly described peripheral innervation of cerebral blood vessels as fibers of peripheral neurons. He returned to the hypothesis that central nervous system neurons in their highly differentiated state would be unable to repeat early developmental processes. Probably due to its high plausibility, this hypothesis persisted until 1980, when A. Aguayo and his collaborators started an in-depth investigation of the role of peripheral nerve transplants as substrates for regenerating central nervous system axons. Their elegant, systematic studies showed that the old findings by Tello were correct and that probably the large majority of central nervous system nerve cells, including projection neurons as well as interneurons, are able to regenerate a lesioned axon in the environment of a peripheral nerve transplant (Richardson et al., 1980, 1984; David and Aguayo, 1981; Carter et al., 1989). Brainstem and spinal cord as well as retinal axons could be guided over distances of several centimeters by Schwann cells and their basement membranes. In recent studies this group showed that retinal axons guided to one of their main target areas, the superior colliculus, through a peripheral nerve graft established typical retinotectal arborizations in the correct layers with synapses closely resembling the normal retinotectal synapse type (Carter et al., 1989, 1991). Typical postsynaptic potentials could be recorded in the deep tectal layers upon light stimulation of the retina (Keirstead et al., 1989). In a related experiment where retinal axons were guided to the pretectal nuclei, a pupilloconstrictor reflex reappeared 6 to 8 weeks after transplantation

(Thanos, 1992). Thus, adult retinal ganglion cells not only possess the capability to regrow long axons, but they obviously retain the mechanisms necessary for target recognition and establishment of functional synapses. Interestingly, however, the fibers leaving the peripheral nerve graft invaded the central nervous system tissue only for a very limited distance of 0.6 mm at most (David and Aguayo, 1981; Carter, 1989; Thanos, 1992). Subsequent investigations of the cell biological mechanisms that could be responsible for this drastic difference between central and peripheral nervous tissue in their properties for neuronal regeneration led to a new concept, that of neurite growth inhibitors (Schwab and Thoenen, 1985; Schwab, 1990), and to the finding that oligodendrocytes are crucially involved in the expression of such molecules (Schwab and Caroni, 1988).

CENTRAL NERVOUS SYSTEM WHITE MATTER AS A CULTURE SUBSTRATE SHOWS NONPERMISSIVE PROPERTIES FOR NEURITE GROWTH

Schwann cells in peripheral nerve enhance the synthesis of neurotrophic factors as well as that of a number of growth-promoting cell adhesion and extracellular matrix molecules in response to denervation (Daniloff et al., 1986; Heumann et al., 1987; Martini and Schachner, 1988; Martini et al., 1990; Meyer et al., 1992). This finding strongly supported the original postulates by Tello (1911) and Ramón y Cajal (1928). However, the situation was much less clear with regard to the central nervous system. We therefore decided to directly test the role of neurotrophic factors *in vitro* by coculturing neurons with explants of adult rat sciatic nerves and optic nerves (central nervous system tissue) in the presence of saturating amounts of neurotrophic factors (Schwab and Thoenen, 1985; Caroni et al., 1988). The transplants were either viable or deprived of living cells by freezing. Interestingly, the results obtained closely corresponded to the *in vivo* situation: sympathetic, dorsal root ganglion, or retinal fibers grew into the sciatic nerve explants, but did not invade the optic nerves. We therefore concluded that neurotrophic factors (nerve growth factor or partially purified brain-derived neutrophic factor) were unable to provoke neurite ingrowth into central nervous system tissue. Instead, these results strongly suggested the presence of inhibitory components restricting neurite growth in central nervous system tissue. Since the same results were obtained with living or dead optic nerves, these components did not arise under the culture conditions but were probably constituents of the adult central nervous system.

Using frozen sections as a culture substrate for various types of neurons growing under neurite-promoting culture conditions Carbonetto et al. (1987) found a very similar result: peripheral nerve tissue was a favorable substrate, but optic nerves or spinal cord tissue did not support neurite growth. In subsequent experiments several groups confirmed this result and showed that in the central nervous system lack of cell adhesion and neurite growth was mainly associated with white matter (Crutcher, 1989; Savio and Schwab, 1989; Watanabe and Murakami, 1889; Khan et al., 1990; Sagot et al., 1991). (However, in comparison to peripheral nerves or artificial growth-promoting substrates, gray matter is not a good substrate either). Because peripheral nerves contain basement membranes around each axon/Schwann cell unit, which were shown to be preferred substrates for regenerating axons *in vivo* (Ide et al., 1983; Schwab and Thoenen, 1985), more detailled cellular and biochemical investigations on the localization of these hypothetical neurite growth inhibitory factors were needed.

IN VITRO INTERACTIONS OF NEURITES WITH GLIAL CELLS

Central nervous system glial cells were cultured at low cell density and characterized by a variety of marker antibodies. The addition of neurons to these cultures revealed large differences in the substrate properties of astrocytes and oligodendrocytes (Schwab and Caroni, 1988). Whereas neuronal cell bodies and, in particular, processes were frequently associated with astrocytes, oligodendrocytes, and the territory covered by their processes seemed to be strictly avoided. A number of recent studies have shown that astrocytes are a less homogeneous cell population than previously assumed, in particular also with regard to their expression of cell surface molecules and extracellular matrix constituents (Hatten et al., 1984; Smith et al., 1986; Grierson et al., 1989; Snow et al., 1990; McKeon et al., 1991). Whereas cultured astrocytes dissociated from embryonic or early postnatal central nervous system are generally excellent substrates for neurite growth, these favorable properties decline with progressive age (Smith et al., 1986, 1990; McKeon et al., 1991). Dense three-dimensional astrocyte networks *in vitro* are not penetrated by postnatal dorsal root ganglion neurites and may resemble the glial scars forming at central nervous system lesion sites (Fawcett et al., 1989a). As these cellular and molecular properties of astrocytes are discussed in other chapters of this book, we will restrict ourselves to oligodendrocytes.

Observations with dissociated cells showed the astonishing result that oligodendrocytes occupied neurite-free areas in dense neuritic networks in culture (Hatten et al., 1984; Schwab and Caroni, 1988; Fawcett et al., 1989b). Importantly, this phenomenon was restricted to differentiated oligodendrocytes ($GalC^+$). $A2B5^+$ precusor cells were often overgrown by neurites (Schwab and Caroni, 1988). Neurons responding by avoidance to oligodendrocytes were ganglion cells from dorsal root or sympathetic ganglia, retinal cells, neuroblastoma cells, and PC12 cells (Schwab and Caroni, 1988; B. Rubin and M. Schwab, unpublished results). Interestingly, neurites from goldfish retinal cells also responded to rat oligodendrocytes by growth arrest, and the oligodendrocyte effect extended also to nonneuronal cells like fibroblasts (Schwab and Caroni, 1988; Bastmeyer et al., 1991).

A detailed time-lapse study was conducted on the interaction between growth cones from rat dorsal root ganglia and oligodendrocytes derived from young postnatal rat optic nerves (Bandtlow et al., 1990). Interestingly, growth cone movement was completely arrested as soon as the tips of a few filopodia had contacted the oligodendrocyte processes. This contact led to retraction of lamellipodia and filopodia, collapse of the growth cone, and growth arrest over many hours. Astrocytes present in the same cultures were crossed at velocities similar to that on the laminin substrate.

All these observations led to the conclusion that oligodendrocytes are a good candidate for growth inhibitory effects in the adult central nervous system.

MEMBRANE PROTEINS OF CENTRAL NERVOUS SYSTEM MYELIN EXERT STRONG NEURITE GROWTH INHIBITORY EFFECTS

Central nervous system myelin was purified from adult rat spinal cord or brain by sucrose density gradient centrifugation, adsorbed to tissue culture dishes, and assayed for its substrate properties for dissociated dorsal root ganglion neurons, neuroblastoma cells, or 3T3 fibroblasts (Caroni and Schwab, 1988a) or adult rat retinal cells (Vanselow et al., 1990). Whereas survival of the cells was unaffected, all types of cells adhered poorly to the myelin substrate, and fiber outgrowth was severely inhibited. Fibroblasts remained round over several hours. Control substrates included peripheral nerve myelin and plasma membranes from liver. Both of these substrates promoted neurite outgrowth and rapid fibroblast spreading. Likewise, myelin of trout or goldfish spinal cord exhibited favorable substrate properties for neurite growth and cell spreading. The inhibitory substrate effect of central nervous system myelin was not extractable by organic solvents, that is, not associated with lipids. Trypsin treatment, in contrast, completely destroyed the inhibitory effect (Caroni and Schwab, 1988a). Removal of peripheral membrane proteins by treatment with high salt or pH 11 did not lead to solubilization of the activity. Rather, detergents like sodium dodecyl sulfate (SDS) or Chaps were required to solubilize the activity. Protein constituents with very similar properties were also found in bovine and human spinal cord myelin (C. Bandtlow and M. Schwab, unpublished observations). Separation of myelin proteins by SDS polyacrylamide gel electrophoresis (PAGE) followed by elution of the proteins, their reconstitution in artificial liposomes and subsequent bioassays on neurons and fibroblasts gave evidence for two main molecular weight fractions exhibiting inhibitory activity at 35 and 250 kD. Immunological data indicate molecular relationships between these two molecular weights (Caroni and Schwab, 1988b). Current work tries to fully purify these components and to obtain enough amino acid sequence information to be able to clone the corresponding cDNAs. Premilinary evidence from limited amino acid sequences points to novel proteins.

It will be of great interest to compare these oligodendrocyte proteins to other repulsive or inhibitory factors that have been characterized in particular in embryonic chicken brain (Raper and Kapfhammer, 1990), early embryonic chicken somite (Davies et al., 1990), and to the putative guidance molecule on posterior tectal membranes of E6–E12 chick embryos (Stahl et al., 1990). Whereas the cell types expressing these molecules are clearly not oligodendrocytes and their biological properties differ from the long-lasting paralyzing action of the myelin proteins, common motifs in the structure of these molecules might occur, and it is possible that more family members of neurite repulsing and neurite growth inhibitory molecules may be found.

Antibodies were raised against the SDS-PAGE purified 35 kD and 250 kD proteins, and monoclonal antibodies were selected for their ability to neutralize the inhibitory substrate action of central nervous system myelin (Caroni and Schwab, 1988b). Monoclonal antibodies (mAB IN-1 and IN-2) were found that strongly reduced the inhibitory action of living oligodendrocytes in the encounter with neurites (Bandtlow et al., 1990), as well as the effect of myelin substrates or myelin liposomes on neurite growth, fibroblast spreading, or growth cone collapse (Caroni and Schwab, 1988b; Bandtlow et al., 1993). When optic nerve explants were injected with

the antibody IN-1, large numbers of DRG neurites grew into the explants over many millimeters.

CELLULAR MECHANISMS OF GROWTH INHIBITION

The sequence of morphological events described above suggests a reorganization of the cytoskeleton with a long-lasting inactivation of the growth cone movement machinery. A similar event might happen in lamellipodia of fibroblasts. Very little information is available at present on these processes. The amount of purified inhibitory protein required to induce growth cone collapse is very low, suggesting specific interactions with putative neuronal membrane receptors. Calcium has recently been investigated as a possible constituent of the intracellular, postreceptor signal cascade. Indeed, fura-2 imaging showed an early, massive calcium increase in DRG growth cones upon contact with liposomes containing the purified 35 kD myelin protein (Bandtlow et al., 1993). Both calcium increase and subsequent growth cone collapse, were prevented by preincubation of the liposomes with mAB IN-1. Interestingly, calcium channels blockers had no effect on this calcium increase. In contrast, Dantrolene, a drug preventing the release of calcium from chaffein-sensitive intracellular stores, prevented growth cone collapse after treatment with 35 kD liposomes (Bandtlow et al., 1993). These results suggest that an intracellular calcium release is crucially involved in the collapse of rat DRG growth cones upon contact with myelin-associated inhibitory proteins. This finding is in line with the calcium homeostasis model predicting that very high intracellular calcium concentrations result in growth cone collapse and growth arrest (Kater and Mills, 1991).

Recent evidence from M. Fishman's laboratory indicated that pertussis toxin, which interferes with the action of certain G proteins, is able to block the effect of central nervous system myelin and purified 35 kD protein (Igarashi et al., 1993). On the other hand, Mastoparan, an activator of G_i/G_o proteins, leads to growth cone collapse, suggesting that G proteins are involved in this signal cascade.

OLIGODENDROCYTE INHIBITORY PROTEINS APPEAR SIMULTANEOUSLY WITH DIFFERENTIATED OLIGODENDROCYTES IN CENTRAL NERVOUS SYSTEM DEVELOPMENT

Inhibitory activity that can be neutralized by the mAB IN-1 was found in rat optic nerves and spinal cord from the time of birth on (Caroni and Schwab, 1989). This time point exactly corresponds to the appearance of $GalC^+$ oligodendrocytes in the optic nerve. Interestingly, however, compact myelin and its main constituents, proteolipid protein and myelin basic protein, are only formed about 1 week later in the optic nerve. The time of appearance of inhibitor proteins also correlates with the end of axon growth in the optic nerve (for references see Caroni and Schwab, 1989). These findings speak in favor of a neurite growth inhibitory function of oligodendrocytes *in vivo*. Immunohistochemical studies of various regions of the adult rat central nervous system showed that IN-1 antigenicity is localized to myelin in white matter, myelinated segments of axons in gray matter, and to oligodendrocytes (Rubin, et al., 1994).

OLIGODENDROCYTE-ASSOCIATED INHIBITORS CAN ACT AS BOUNDARY MOLECULES FOR LATE GROWING CENTRAL NERVOUS SYSTEM FIBERS

In a given central nervous system region, myelin formation is known to occur in a mosaiclike pattern with high tract and regional specificity. Thus, various fiber tracts or nuclear areas in the brainstem or in the spinal cord myelinate at precise time points within the first 2 postnatal weeks in the rat (Rozeik and von Keyserlingk, 1987; Schwab and Schnell, 1989). The period of myelination in the early myelinating tracts overlaps with the period of axon growth in late-developing fiber systems. The possibility, therefore, exists that oligodendrocyte-associated neurite growth inhibitory proteins serve as "no-go" signals in certain anatomical structures. This hypothesis was investigated in two systems, the corticospinal tract in the spinal cord (Schwab and Schnell, 1991), and the termination layers of the retinal fibers in the superior colliculus (Kapfhammer et al., 1992).

Corticospinal tract axons grow into the rat spinal cord at birth and complete their growth around postnatal day 10 (P10). Most of the fibers grow in the dorsal funiculus, adjacent to the ascending sensory fasciculus cuneatus and gracilis. These ascending tracts start myelination in the cervical spinal cord around day P1. It was interesting to see that a very sharp border exists between the corticospinal tract and the sensory tracts in the cervical and upper thoracic spinal cord, whereas this border was less precise in the lower half of the spinal cord (Schwab and Schnell, 1991). This pattern corresponded exactly to the presence of differentiated oligodendrocytes in the sensory tracts in the rostral half of the spinal cord and their absence in the caudal half of the spinal cord at the time of corticospinal tract fiber ingrowth. Two

experimental paradigms were used to further test the hypothesis of negative guidance by oligodendrocyte-associated inhibitory proteins: oligodendrocyte development was prevented by neonatal x-irradiation of the spinal cord, or, alternatively, mAB IN-1 was applied to newborn rats. In both cases, tracing of the corticospinal tract resulted in identical anatomical abnormalities: (1) the sharp border between corticospinal tract and ascending sensory tracts in the rostral spinal cord was absent, and (2) outgrowth of collaterals into the gray matter occurred prematurely. These results show that the development of tracts at different times can lead to anatomical separation by the expression of oligodendrocyte-associated inhibitory proteins. In addition, oligodendrocytes seem to be able to restrict or delay the outgrowth of collateral branches.

Destruction of the upper layers of the *superior colliculus of hamsters* at birth leads to an ingrowth of retinal axons into the contralateral optic tectum (So and Schneider, 1978). Interestingly, these fibers do *not* grow in a pattern appropriate for retinal axons; they completely avoid the optic fiber layer (stratum opticum) and only innervate the most superficial part of the stratum grisium superficiale. Since these axons reach the optic tectum about 5 days later than the normal retinal axons, they encounter differentiating oligodendrocytes in the stratum opticum (Jhaveri et al., 1992). The mAB IN-1 was therefore applied at the time of operation (Kapfhammer et al., 1992). This resulted in the restoration of a seemingly normal anatomical pattern; the recrossing retinal fibers now grew and terminated in both, the stratum opticum and the stratum grisium superficiale. As the superior colliculus is a cortical structure, these results show that the oligodendrocyte-associated IN-1 antigents can also be involved in closing down neurite growth in particular layers of cortical brain structures at given developmental times.

NEUTRALIZATION OF INHIBITORY PROTEINS *IN VIVO* ENHANCES REGENERATION OF TRANSECTED AXONS IN SPINAL CORD AND BRAIN

In the studies on *spinal cord* tract we again concentrated on the corticospinal tract due to its large number of fibers, its defined localization, and the ease with which this tract can be labeled with the sensitive anterograde tracer wheat germ agglutinin-horseradish peroxidase (WGA-HRP). Oligodendrocyte-associated inhibitory activity was compromised in two different ways: by deleting the dividing oligodendrocyte precursors by high-dose x-irradiation (Savio and Schwab, 1990), or, alternatively, by the application of mAB IN-1 (Schnell and Schwab, 1990, 1993). Complete bilateral transections of the dorsal two-thirds of the spinal cord were done in the thoracic region, and regeneration was studied at 2 to 12 weeks of survival. Animals were young (2 to 4 weeks) or fully adult (12 weeks) at the time of the lesion; regeneration of corticospinal tract axons has been shown to be completely absent at these ages in earlier studies (Bernstein and Stelzner, 1983). This finding was confirmed in all our controls, including rats with control antibodies (Figure 57-1). Interestingly, almost identical results were obtained with the two experimental paradigms: in the absence of oligodendrocytes and myelin, or in the presence of the

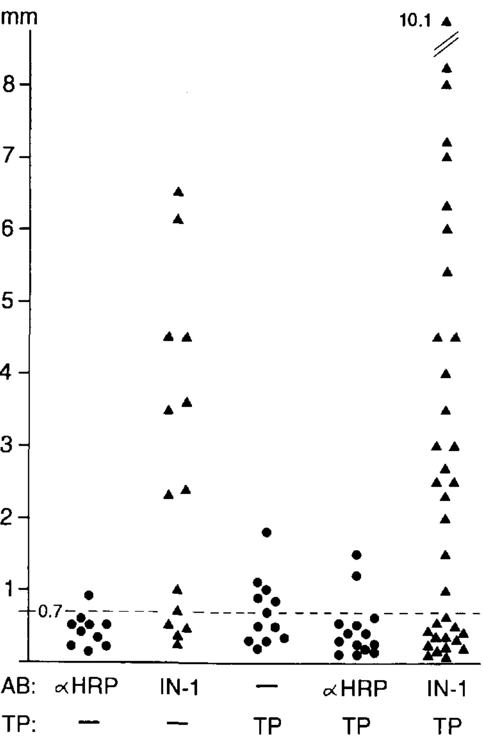

FIG. 57-1. Maximal distances of corticospinal tract (*CST*) elongation in presence or absence of an E14 spinal cord transplant (*TP*) and the antibody (*AB*) IN-1 directed against the oligodendrocyte-associated neurite growth inhibitors, or a control *AB* (anti-HRP). Each symbol represents the maximal regeneration distance of an individual rat. *Dashed line:* sprouting distance of 0.7 mm. Camera lucida drawings of two controls and two IN-1 animals are shown Figure 57-2A–D, and darkfield micrographs of regenerating fibers in Figure 57-3A–F. *Methods:* The dorsal half of the spinal cord was transected bilaterally at the lower thoracic level in 3- to 4-week-old rats. Some of the animals simultaneously received a transplant of embryonic day 14 (*E14*) spinal cord tissue at the lesion site. Control (anti-HRP) or IN-1 antibodies were applied as hybridoma tumors transplanted to the left parietal cortex. The corticospinal tract was traced by anterograde transport of WGA-HRP from the right sensory motor cortex 2 to 3 weeks after the lesion. [From Schnell and Schwab (1993), with permission.]

inhibitor-neutralizing antibody IN-1 a small percentage of corticospinal tract axons were found to elongate from the lesion site over several millimeters toward the caudal end of the spinal cord (Savio and Schwab, 1989; Schnell and Schwab, 1990, 1993). Great variations existed from animal to animal in the distance of regeneration (Figure 57-1). However, in most of the successfully regenerating animals distances of 4 to 6 mm were obtained (Figures 57-1, 57-2, and 57-3). The best cases showed regenerations which reached the lower lumbar and sacral spinal cord. The relatively small number of regenerating fibers could be due to several factors: in the antibody-treated animals, penetration of antibodies into the central nervous system tissue was quite variable and, at longer distances from the lesion site, very limited.

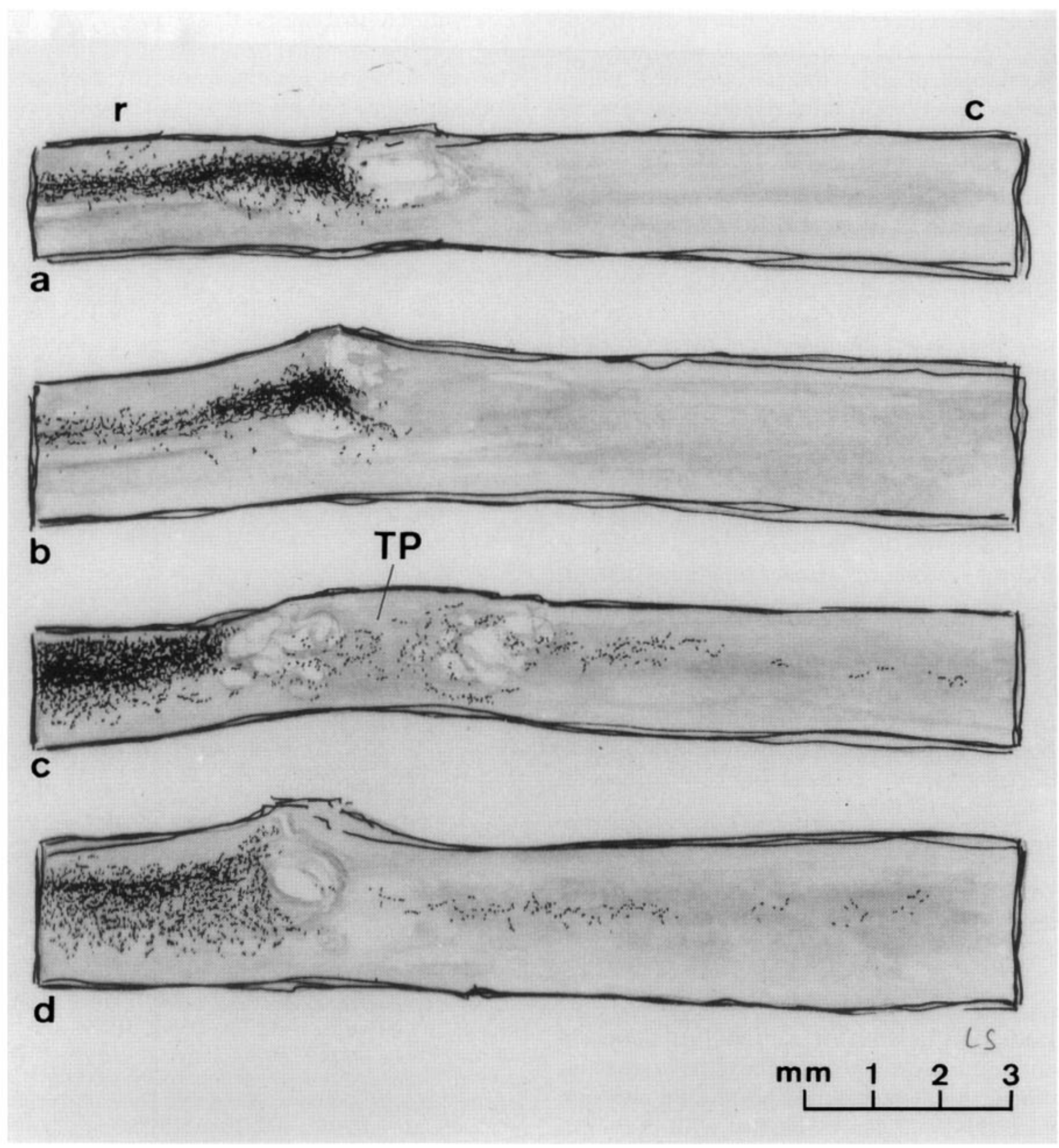

FIG. 57-2. Camera lucida drawings. (**A, B**) Consecutive, parasagittal sections of two control antibody (anti-HRP)-treated rats. (**C, D**) Two IN-1 antibody treated rats. All animals (except Figure B) possess embryonic days 14 to 16 spinal cord transplants (*TP*). Regenerative growth of corticospinal tract fibers over long distances is present exclusively in the IN-1-treated rats; 6 to 8 consecutive sections were compiled and projected on top of each other. Note the typical irregular course of the regenerated fibers. *r*, rostral; *c*, caudal; *m*, medial; *l*, lateral aspect of the spinal cord; *blank areas,* lesion caverns.

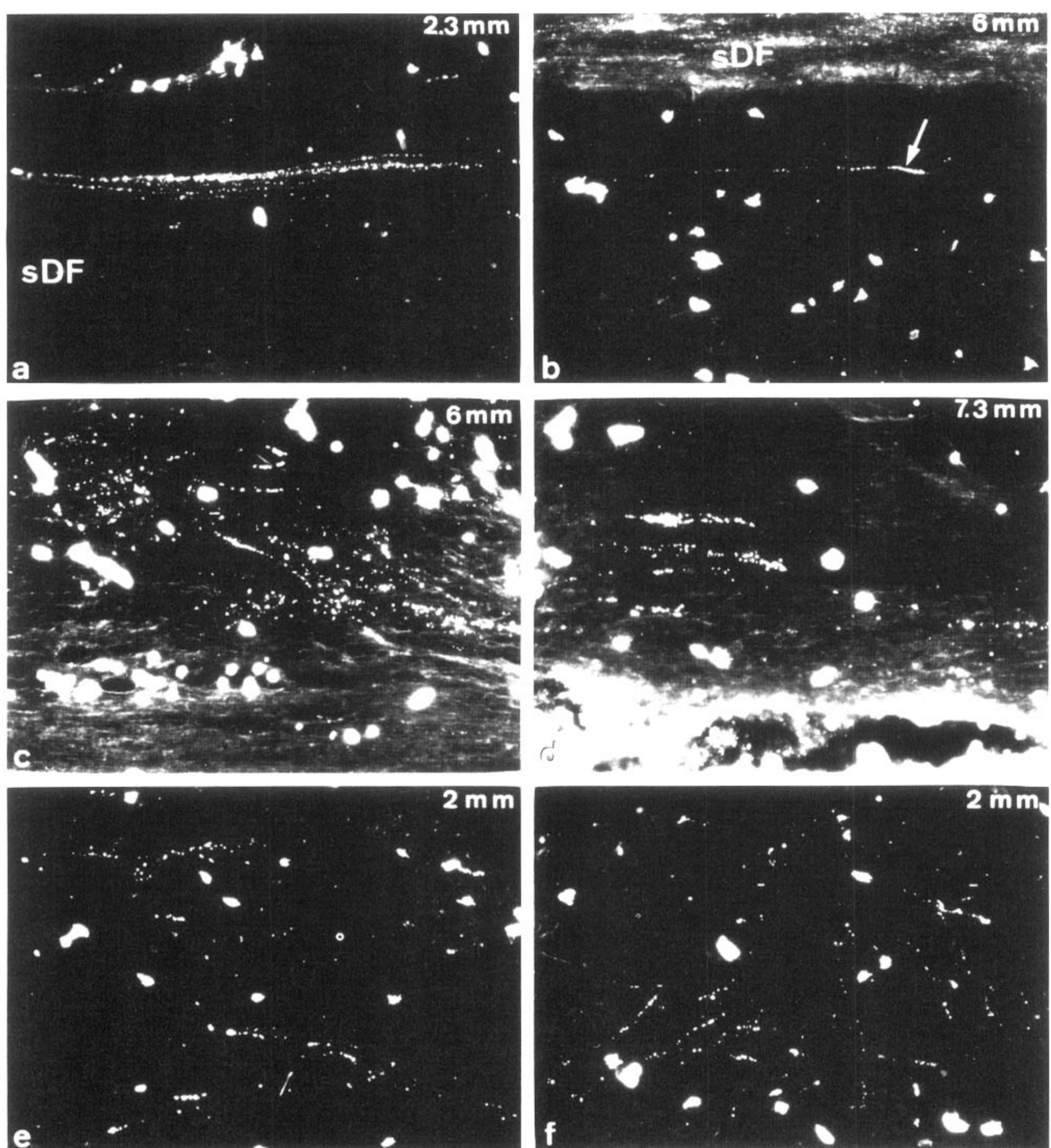

FIG. 57-3. High magnification darkfield micrographs of regenerating corticospinal tract fibers at 2 to 7.3 mm caudal of the lesion. (**A**) Fascicle of regenerating fibers in the sensory tracts of the dorsal funiculus (*sDF*). (**B**) Single axon with growth cone in the original corticospinal tract position ventral to the sensory tracts of the dorsal funiculus. (**C**) Fibers and fascicles in gray matter, (**D**) dorsolateral (*upper bundle*) and ventral funiculus of the same rat at 6 and 7.3 mm from the lesion. (**E, F**) Branching fibers in gray matter. Rats were lesioned at 3 to 5 weeks and survived for 2 to 4 additional weeks. ×120.

In addition, the lesion area itself was clearly an obstacle to regenerating fibers. Often, the majority of the regenerative sprouts were unable to cross the lesion area and failed to reach the caudal end of the lesion site (Figure 57-1). Almost all of the crossing fibers used intact spinal cord tissue (mostly ventral gray and white matter) as a bridge. Thus, it is not excluded or even probable that additional inhibitory factors occur at these lesion sites, which impair the crossing of this area by regenerating fibers. An additional factor is the regenerative sprouting itself, which, in the case of the corticospinal tract, is not very robust. Experiments with transplants of embryonic spinal cord tissue and recent experiments with trophic factors indicate that this sprouting response can be significantly enhanced (Schnell and Schwab, 1993; Schnell et al., 1993). Interestingly, however, the regenerative sprouts did not exceed distances of more than 0.7 to 1 mm unless the antibody IN-1 was present (Figures 57-1, 57-2, and 57-3).

Using IN-1 antibodies in *fimbria fornix* ablation lesions we could show that the reinnervation of the hippocampus by septal cholinergic axons is accelerated by this antibody (Cadelli and Schwab, 1991). At 2 to 5 weeks after the lesion and the implantation of a 2-mm-long extracellular matrix bridge, regenerated fibers could be found preferentially in their former target areas. These findings show that IN-1 antigens severely impair regeneration also in the hippocampus and in gray matter areas.

Preliminary results in rat *optic nerves* closely resemble results obtained in spinal cord. Deletion of oligodendrocytes by x-irradiation or application of the antibody IN-1 following an intracranial crush lesion led to regeneration of axons over a distance of several millimeters.

The paralellisms of the results of the two experimental paradigms studied, and the fact that a monoclonal antibody directed against 35/250 kD inhibitory proteins of oligodendrocytes had such a drastic effect under *in vivo* conditions strongly argue for a significant role of oligodendrocytes in restricting central nervous system regeneration. This hypothesis is supported by experiments in chick embryos, which showed that spinal cord tract regeneration stops exactly at the time of the appearance of oligodendrocytes and myelin (Shimizu et al., 1990; Hasan et al., 1991). Deletion of oligodendrocytes by immunocytolysis prolonged the regeneration-permissive period by several days (Keirstead et al., 1992). However, additional negative signals, in particular in lesion areas, as well as important roles for positive substrate molecules, trophic factors, and intrinsic neuronal determinants must be assumed and will form the basis for future investigations.

In conclusion, oligodendrocytes, in addition to their function as myelin-forming cells, also express potent neurite growth inhibitory proteins. These molecules are expressed at the time of myelination and throughout adult life. Their physiological functions may be to inhibit the access of late growing fibers to certain territories during development, and to restrict sprouting and plasticity in the adult. Following central nervous system lesions in rats, significant long distance elongation of fibers was obtained by the neutralization of these inhibitory proteins, or by the deletion of oligodendrocytes. This result suggest an important role of these myelin-associated neurite growth inhibitors in the context of central nervous system regeneration.

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58 Transplantation of glial cells

W. F. BLAKEMORE, A. J. CRANG, AND R. J. M. FRANKLIN

Glial cell transplantation into the central nervous system offers an experimental approach to help unravel the complex interactions that occur between central nervous system glia, Schwann cells, and axons during repair and development. Although glial cell transplantation, in the form of peripheral nerve grafting was carried out by Santiago Ramón y Cajal and Francisco Tello early in this century, it was the development of glial cell culture techniques, and the consequent increase in our understanding of glial cell lineages, that added a new dimension to this experimental paradigm. Labeled glial cells can now be introduced into the developing nervous system to examine aspects of development and glial cell lineage, while transplantation of glial cells into lesion environments yields information about cell-cell interactions in repair, and opens up the possibility of influencing normal cell responses by transplantation. This chapter concentrates on the principles underlying glial cell transplantation and documents some of the results achieved over the last decade.

PRACTICAL CONSIDERATIONS THAT UNDERLIE GLIAL CELL TRANSPLANTATION

As the normal glial population of the central nervous system has an intrinsic replacement capacity, there are two major issues that must be addressed when carrying out and interpreting glial cell transplantation studies. First, one must be able to identify the transplanted cells, either directly in order to study migration and integration into normal tissue, or by using an experimental design that allows identification of the effect of transplantation in a pathological situation. Second, one must have sufficient information about the composition of the transplant to be able to relate the subsequent observed behavior of transplanted cells to the preparation introduced.

Identification of Transplanted Cells

Various strategies have been used to distinguish transplanted cells from host cells and nearly all impose limitations on the type of studies that can be undertaken. Transplanted cells can be labeled with vital dyes such as fast-blue (Rosenbluth et al., 1990; Hasegawa and Rosenbluth, 1991; Hatton et al., 1992) and bisbenzimide (Hoechst dyes 33258 and 33342) (Figure 58-1) (Baron-Van Evercooren et al., 1991; Gansmüller et al., 1991; Tourbah et al., 1991) or fluorochrome-conjugated lectins (Goldberg and Bernstein, 1987; Bernstein and Goldberg, 1989) for subsequent identification using fluorescence microscopy. These labels, like the use of tritiated thymidine, suffer the disadvantage of dilution by repeated division of cells. Thus, following transplantation of highly proliferative populations of cells the intensity of label signal within the progeny may fall below the threshold for detection. With vital dyes care must be taken to wash cells prior to transplantation in order to prevent direct labeling of host tissue by free label present within the transplant preparation. Phagocytic uptake of fluorescent microspheres (Emmett et al., 1988; Hatton et al., 1992) has also been used to label cells but suffers similar dilution problems as the vital dyes. An additional feature of this approach is that microspheres are preferentially taken up by astrocytes and microglia rather than oligodendrocyte lineage cells. This may be deemed an advantage or disadvantage, depending on whether a pan-marker or cell-specific marker is required. With many of the aforementioned labels the possibility exists that host cells may incorporate label liberated from transplanted cells as either a spontaneous process or as a consequence of cell death or damage. This, however, appears not to be a problem with tritiated thymidine where there is no evidence of uptake of thymidine from transplanted cells into host cells (Emmett et al., 1991).

If protocols require identification of transplanted cells and their progeny, there are few suitable markers available. One approach is to use species-specific markers, and these have been successfully used to identify and trace migration of astrocytes following xenografting (Jacque et al., 1986; Zhou et al., 1990). Another approach is to use genetic markers. Genetic markers can be inherent, such as the detection of repeat sequence on the Y chromosome by *in situ* hybridization following transplantation of male cells

FIG. 58-1. Vital stains can be employed to allow the distinction of transplanted cells from host tissue. Here transplanted cells labeled with the Hoechst dye 33342 can be easily detected following transplantation using long-wavelength UV fluorescence excitation of cryostat sections. ×100. [Micrograph kindly provided by M. Gumpel and A. Baron-Van Evercooren.]

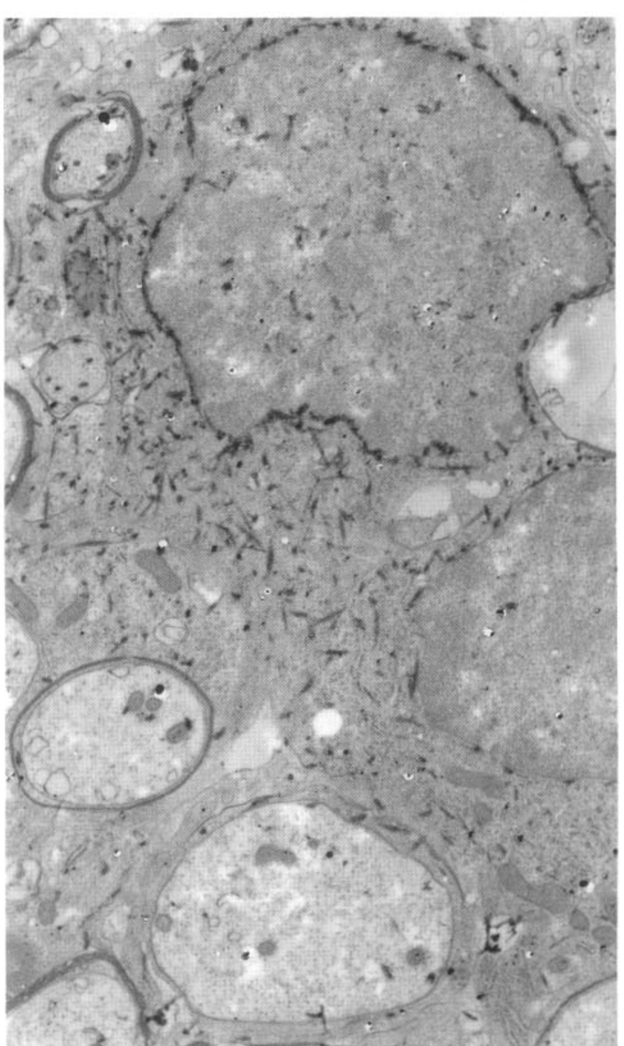
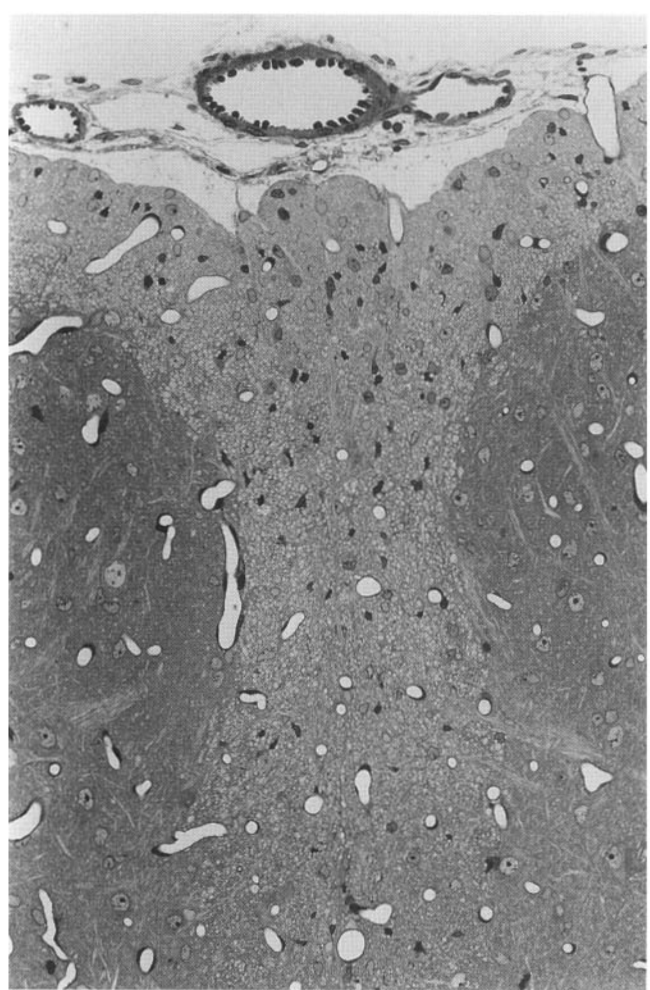


FIG. 58-2. The introduction of foreign genes into cells prior to transplantation in principle allows the identification of all the progeny of a transplanted cell preparation. In this micrograph, rat O-2A progenitor cells expressing *E. coli* β-galactosidase develop into oligodendrocytes following transplantation into an area of demyelination. The β-galactosidase reaction-product is visible as electron-dense spicules within the cytoplasm of the oligodendrocyte and in association with oligodendrocyte processes that have surrounded the demyelinated axons. Unstained section. ×13,000.

into female recipients (Harvey et al., 1992). At present, probes to rat cells are not available. Alternatively, they can be foreign genes present within transgenic animals or introduced into cultured cells by transfection. An example of transgenic-derived donor material is provided by the study of Huppes and coworkers (1992), who transplanted tissue from a transgenic mouse containing multiple copies of the bacteriophage lamda gene. The introduction of reporter genes into cultured cells has also been used successfully to identify transplanted cells (Figure 58-2.) (Langford and Owens, 1990; Groves et al., 1993). This technique has the disadvantage of requiring dividing cells for retroviral insertion and suffers from the problem that not all transfected cells express the gene product. The use of marker genes, such as *LacZ,* under cell-specific promotors, may ultimately provide excellent material for glial cell transplantation studies.

The problems associated with many of the cell-labeling techniques can be overcome by transplanting cells into situations where the simple presence of glial cells or myelin sheaths identifies them as being of transplant origin. In the case of oligodendrocytes, this is achieved by introducing normal cells into myelination-defective mutants, such as the myelin basic protein-deficient Shiverer mouse (Gumpel et al., 1983; Friedman et al., 1986; Kohsaka et al., 1986; Gumpel et al., 1989), or the various proteolipid protein mutants: the myelin-deficient rat (Duncan et al., 1988; Rosenbluth et al., 1990) (Figure 58-3), the Jimpy mouse (Lachapelle et al., 1991) and the shaking pup (Archer et al., 1992). All the proteolipid protein mutants show such severe hypomyelination that myelin formation observed following transplantation can be related to transplanted cells. Immunostaining for proteolipid protein can if necessary be used to distinguish host, proteolipid protein-deficient myelin from the proteolipid protein-positive myelin produced by transplanted cells (Duncan et al., 1988). Myelin basic protein-positive normal myelin



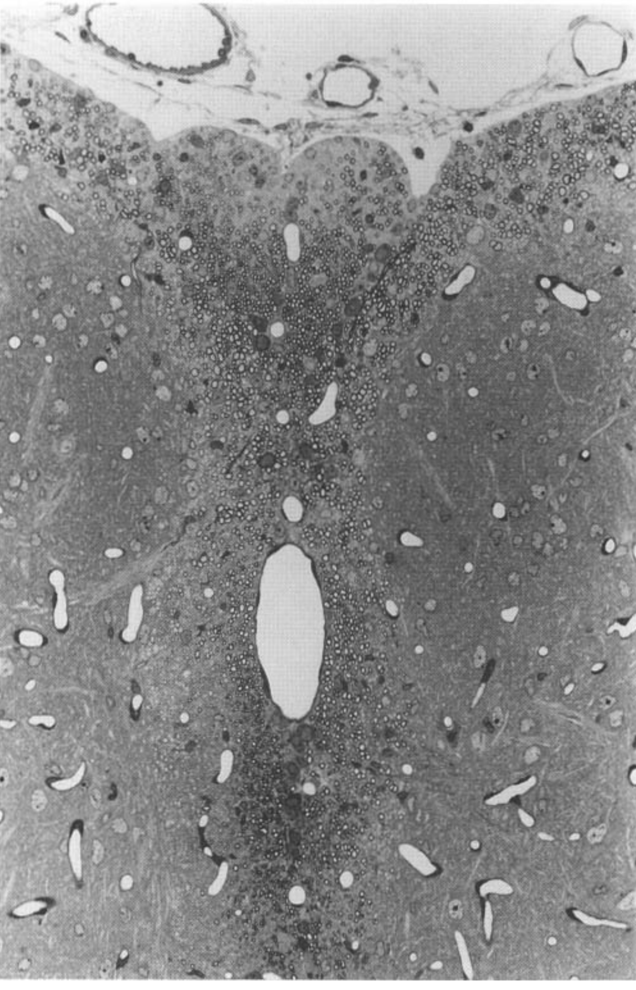


FIG. 58-3. Myelination mutant animals allow the identification of transplanted wild-type oligodendrocytes. (A) The spinal cord of the myelin-deficient rat contains no myelinated axons and so the myelinating potential of transplanted cell preparations can be related to the extent of myelination seen in transplanted animals. (B) At 12 days following the transplantation of a mixed glial cell culture prepared from neonatal normal rats, regions of oligodendrocyte myelination are found. ×220. [Micrographs kindly provided by I. Duncan and D. Archer.]

is easily recognized in the Shiverer mouse either by immunocytochemistry (Lachapelle et al., 1984), or by electron microscopy (Gansmüller et al., 1986) (Figure 58-4). Areas of the central nervous system that are not normally myelinated, such as the retina, also provide a situation where the presence of myelination following transplantation can be related to transplant-derived cells (Huang et al., 1991).

An alternative application of this strategy is to create glial-deficient lesions that have no inherent repair capacity (Blakemore, 1977; Blakemore and Patterson, 1978; Blakemore and Crang, 1988; Crang et al., 1992). This can be achieved by injecting gliotoxins such as ethidium bromide into tissue that has been exposed to 40 Gy of x-irradiation. Following the injection of ethidium bromide into white matter, both oligodendrocytes and astrocytes are intoxicated, and their subsequent death leaves demyelinated axons in a region devoid of central glial cells (Figure 58-5). Such lesions have clearly defined edges that allow areas of remyelination and repair to be easily identified. The essential nonrepairing nature of the lesion can be rigorously tested by showing that the injection of cell preparations shown to recruit cells in normal animals fails to recruit cells from the host when injected into x-irradiated lesions (Blakemore, 1992; Crang et al., 1992). Furthermore, there is a direct correlation between the nature of repair observed and the composition of the cells introduced into this environment. This nonrepairing, cell-free lesion therefore provides a suitable system in which to assess the ability of transplanted glial cells to associate with demyelinated axons and reconstruct central nervous system environments.

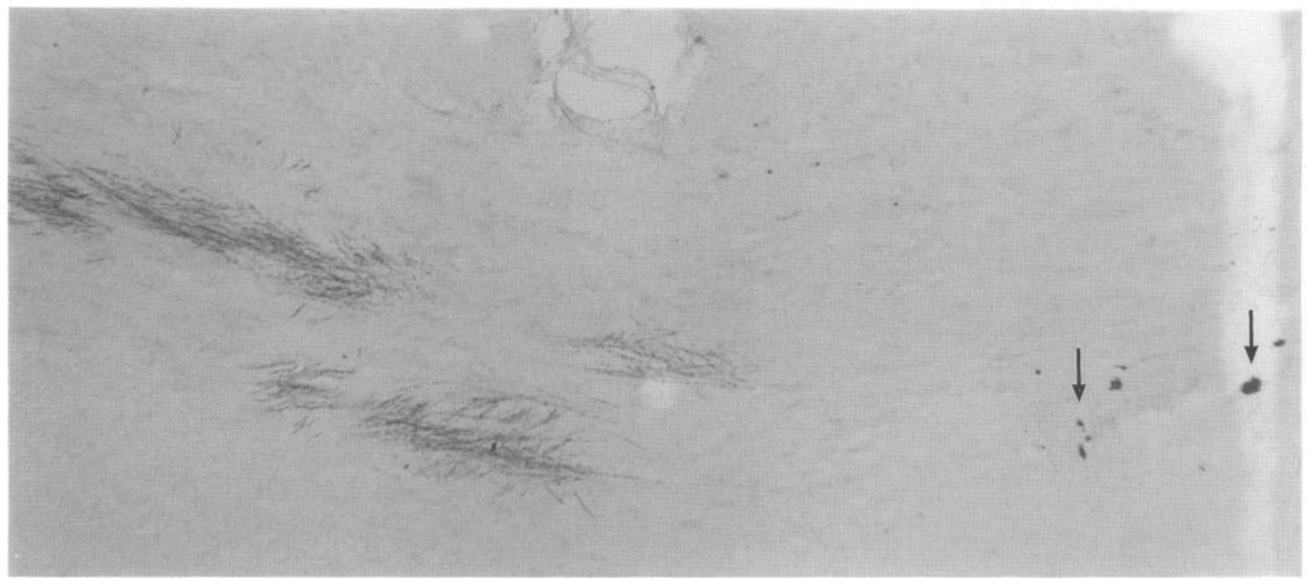


FIG. 58-4. Mice homozygous for the autosomal recessive mutation *shiverer* lack myelin basic protein (*MBP*). In this micrograph, MBP-containing myelin sheaths are detected 23 days after transplantation of a fragment of normal E15 central nervous system tissue into a newborn *shiverer* mouse brain. The two patches of myelination are present at some distance from the point of implantation marked by carbon (*arrows*). ×100. [Micrograph kindly provided by M. Gumpel and F. Lachapelle.]

In pathological situations in which normal glial responses are intact, transplanted cells require labels in order to be individually identified. However, the effects of transplantation can be demonstrated using simple morphological analysis. An example is provided by the ability to transplant glial cells to alter the repair of experimentally induced demyelinating lesions in the central nervous system. Following the injection of ethidium bromide into normal white matter of the spinal cord of the rat, the demyelinated axons are normally remyelinated mainly by Schwann cells (Graça and Blakemore, 1986). However, when mixed glial cell cultures are injected into the lesion, remyelination is mainly carried out by oligodendrocytes and this can be documented quantitatively using plots of Schwann cell versus oligodendrocyte remyelination (Blakemore and Crang, 1989, 1992) (Figure 58-6). Similarly, the ability of astrocyte transplantation to reduce the area of necrosis that follows spinal concussion can be documented by morphological and functional assessment of transplanted and nontransplanted groups of animals (J. Wrathall, personal communication).

Manipulating the Transplant Composition

Most of the early work involving transplantation into myelin mutants addressed relatively straightforward issues, such as whether axons could be myelinated by transplant-derived oligodendrocytes and the area over which this could be achieved. To some extent, the recipient environment provided by the myelin mutants imposes these limitations. For example, the Shiverer mutant is appropriate for studying the oligodendrocyte lineage rather than the astrocyte lineage. Moreover, the precise identification of the cell populations introduced was not a major concern, as both the environment into which the transplant was introduced and the markers used to identify the transplanted cells dictated the questions that could be addressed. Glial cell transplantation studies are now entering a new era in which more exacting questions are being asked. At present, this represents the most challenging area in the field. When tissue fragments are used as the source of transplanted cells it is only possible to analyze and comment on the cells that emerge from the tissue fragment. While this is largely controlled by the environment, a degree of control can be exercised by using fragments obtained from animals of different age (Gumpel et al., 1985; Friedman et al., 1986; Rosenbluth et al., 1990), or brain area (Jacque et al., 1991), or pathological tissue such as degenerated optic nerve (Ludwin, 1992).

When the transplanted tissue has been subjected to tissue culture before transplantation, it is possible not only to identify the cell types that will comprise the transplant but also to subject the cultures to a series of *in vitro* manipulations that will enrich the cell suspension for particular subsets of cell. These

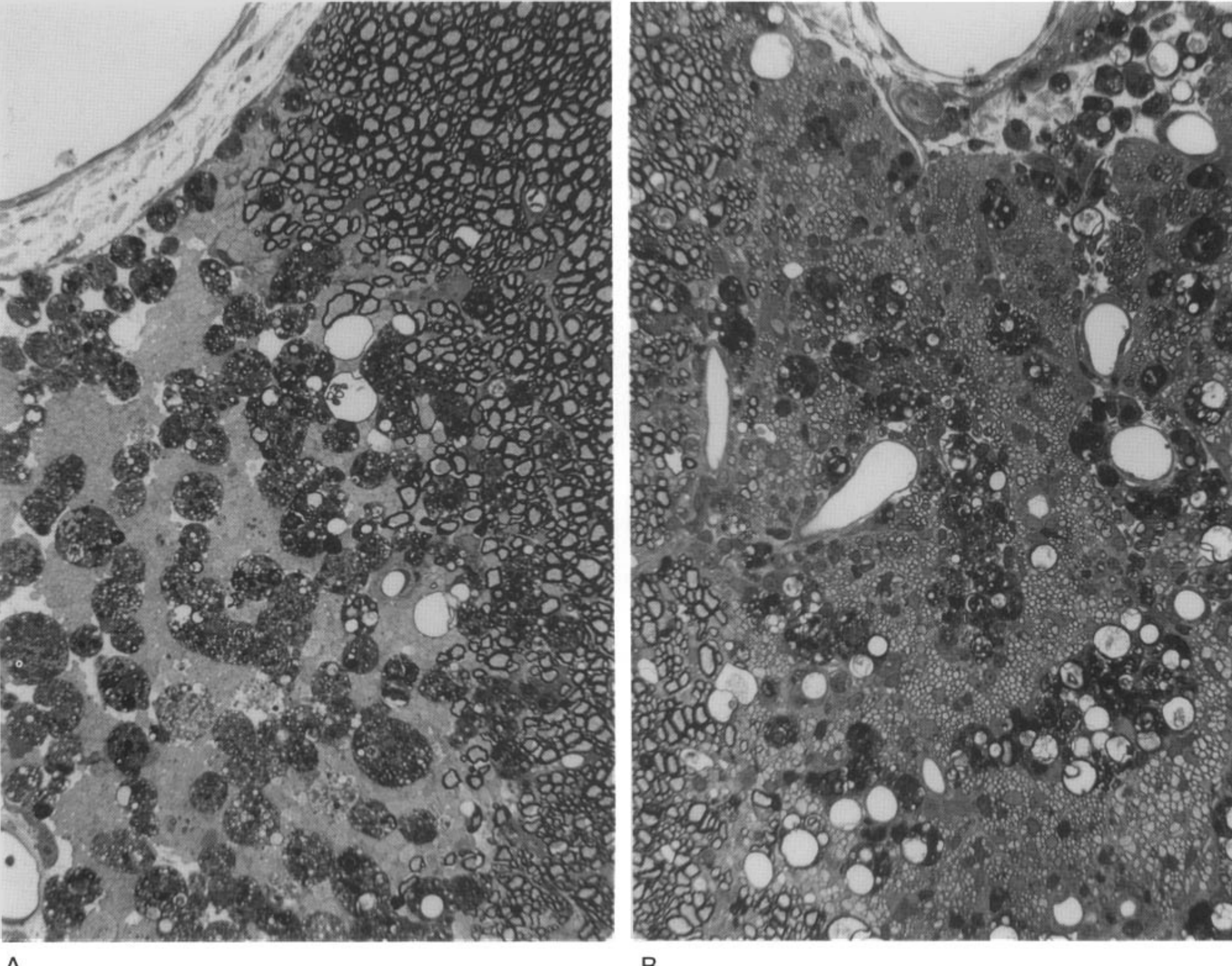


FIG. 58-5. The nonrepairing ethidium bromide white matter lesion provides a situation in which to assess the ability of transplanted glial cells to associate with demyelinated axons and reconstruct central nervous system environments. (**A**) Following the injection of ethidium bromide into an area of spinal cord exposed to 40 Gy of x-irradiation, demyelinated axons clump together in a glial cell-free environment and there is no evidence of remyelination. The lesion contains debris-filled macrophages and has a clearly defined edge. (**B**) Transplantation of cultures of syngeneic mixed glial cells into nonrepairing lesions results in the reconstruction of a normal central nervous system glial cell environment, most of the demyelinated axons being remyelinated by oligodendrocytes. ×340.

procedures include separation of heterotypic mixed glial bilayers (Blakemore and Crang, 1989), depletion by complement-mediated immunocytolysis (Franklin et al., 1991) or x-irradiation (Franklin et al., 1993a), and purification by cell sorting (Duncan et al., 1992) or panning (Warrington et al., 1993). A more recent approach is to obtain pure populations of cells of specific phenotypes by exposure to growth factors (Groves et al., 1993) or by the creation of cell lines of either central glia (Crang et al., 1992; Barnett et al., 1993; Trotter et al., 1993) or peripheral glia (Baron-Van Evercooren et al., 1992a, 1992b, Jung et al., 1994) (see below). The ability to determine the transplant composition with some precision provides the experimenter with the necessary control to address subtle questions about complex cellular interactions. However, the ability to accurately describe the content of a tissue culture derived transplant is limited not only by the range of antibodies available to identify cells in culture, but also by the ability to visualize very small numbers of antibody-labeled cells in high-density cultures. The possibility of unrecognized contaminants even within well characterized cell preparations must be taken into account when interpreting the effects of transplantation, since such cells can become, or give rise to, major populations in certain transplanted environments (Blakemore et al., 1987b; Crang and Blakemore, 1989; Franklin et al., 1992a, 1992b, 1993a).

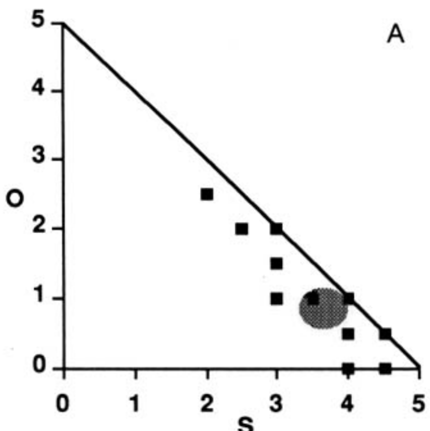


FIG. 58-6. The nature and extent of the repair of spinal cord white matter ethidium bromide lesions can be documented quantitatively by using plots of oligodendrocyte (*O*) versus Schwann cell (*S*) remyelination. The proportion of axons remyelinated by oligodendrocytes and Schwann cells, and the proportion of axons that remain demyelinated are estimated for 1-μm plastic sections cut from each lesion-containing block (see Blakemore and Crang, 1992). Thus, a section in which all the available axons are remyelinated by oligodendrocytes would have an oligodendrocyte remyelination score of 5, a Schwann cell remyelination score of 0, and a demyelinated axons score of 0. The mean oligodendrocyte and Schwann cell remyelination scores are indicated ±2 SEM and the shaded domains enclosed by these limits represent an average repair for the group of animals. Nonoverlapping repair domains in these representations therefore indicate significantly different results; the direction of their displacement from each other indicates which parameters contribute to the difference. (**A**) Following the injection of ethidium bromide into spinal cord white matter, the demyelinated axons are remyelinated mainly by Schwann cells. (**B**) When mixed glial cell cultures are injected into the demyelinating lesion, remyelination is mainly carried out by oligodendrocytes.

Migration Studies

Migration of glial cells following transplantation can not be followed directly, but it can be inferred by killing animals at different survival times and plotting the expanding distribution of suitably labeled cells (see above for constraints of cell labeling techniques). From these "snapshots" the rate and extent of migration of cells can be established. In order to obtain a true picture of the extent to and rate at which cells migrate, it is necessary to know the limits to which the cells are distributed by the physical process of transplantation alone. When cells are transplanted as a cell suspension, this may be highly variable and difficult to assess. Thus, the precise point of injection at which cells start to migrate cannot be easily established. For this reason the most reliable studies on cell migration have involved transplanting cells within tissue fragments (Lachapelle et al., 1984), or within aggregates (Emmett et al., 1991).

WHAT HAVE TRANSPLANTATION STUDIES TOLD US ABOUT THE BIOLOGY OF GLIAL CELLS?

Oligodendrocytes

Transplantation of cells of the oligodendrocyte lineage into neonatal recipients has been used to study migration of myelin-forming cells and to examine concepts about migration and differentiation that have emerged from *in vitro* studies on cell lineage and development. For technical reasons these studies have been carried out using the myelin mutants. The initial studies in the Shiverer model using fragments of central nervous system tissue, as the source of myelin-forming cells, showed that cells migrated long distances from the site of implantation before differentiating into myelin-sheath-forming oligodendrocytes (Gumpel et al., 1983; Lachapelle et al., 1984). Recent studies using Hoechst dye labeling and immunocytochemical identification of cells (Gansmüller et al., 1991), or immunopurification of the transplanted cells (Warrington et al., 1993), have demonstrated that oligodendrocyte precursor cells migrate and then divide at the site of terminal differentiation, with the more immature phenotypes giving rise to the largest areas of myelination. Transplantation of O1+ cells (Warrington et al., 1993), or of cells that persist in areas of gliosis following axon degeneration (Ludwin, 1992), result in only small areas of myelination, located close to the site of transplantation. A start has been made to examine the migration of cells toward areas of demyelination in adult animals. These studies indicate that cells migrate from fragments of transplanted tissue toward areas of demyelination (Gout et al., 1988;

Gumpel et al., 1989), in contrast to random migration if no lesion is present.

In the Shiverer mutant, the transplanted cells have to compete with host cells for axons as they migrate within a myelinating environment. In the myelin-deficient rat, there is little if any host myelination, and long-distance migration of cells has not so far been observed (Rosenbluth et al., 1990). It is thus an ideal model for determining the areas of tissue that can be populated by transplanted cells. Using this mutant it has been shown that the age of the transplanted cells or central nervous system fragment can be directly related to the extent of myelin sheath formation (Rosenbluth et al., 1990; Archer et al., 1993); little or very restricted myelin sheath formation is seen following transplantation of material from adult animals. This observation confirms earlier studies made using the Shiverer model (Gumpel et al., 1985; Friedman et al., 1986). However, in a recent study, extensive myelination was claimed following transplantation of FACS purification of O1+ cells obtained from adult animals (Duncan et al., 1992). It is likely that further studies will be needed before a full understanding of the myelinating potential of cells from the adult nervous system is accomplished.

The shaking pup deserves special mention, as this is the only large animal in which extensive myelination has been achieved following transplantation. Using these animals Duncan and Archer have shown that extensive migration (up to 3 cm) and myelination was present 11 weeks after transplantation of fetal tissue into the spinal cord of a 14-day-old pup (Archer et al., 1992; I. D. Duncan, personal communication). No evidence of rejection was observed.

A series of cross-species studies have been carried out, demonstrating that oligodendrocytes from one species will form myelin sheaths around axons of another species, that is, mouse (Crang and Blakemore, 1991), cat (unpublished observations), and dog (Archer et al., 1994) will myelinate rat axons; rat (Lubetzki et al., 1988), sheep (Ludwin and Suchet, 1993), and human (Gumpel et al., 1987) will myelinate mouse axons; and rat will myelinate cat axons (unpublished observations). Most of the xenografting experiments have been carried out in neonatal animals and in these circumstances no evidence of rejection has been reported. However, when mouse and cat cells are introduced into demyelinating lesions in adult rats, high levels of immunosuppression are needed to prevent rejection (Crang and Blakemore, 1991) (Figure 58-7), and transplanted xenogeneic cells are rapidly rejected on removal of immunosuppression (Blakemore, 1992).

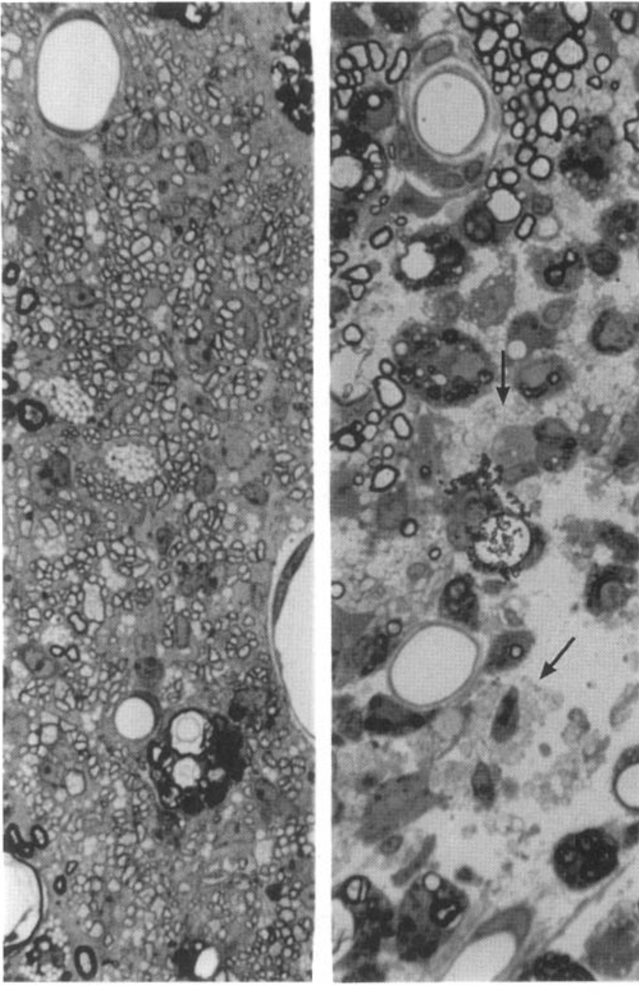


FIG. 58-7. When xenogeneic glial cells are transplanted into demyelinating lesions in adult rats, immunosuppression is required to prevent rejection. (**A**) Following transplantation of mouse mixed glial cell cultures into x-irradiated ethidium bromide lesions in rats, reconstruction of a central nervous system glial environment only occurs in immunosuppressed hosts. ×410. (**B**) In nonimmunosuppressed rats, lesions transplanted with mouse mixed glial cell cultures show no evidence of repair and demyelinated axons (*arrows*) exist in areas infiltrated by inflammatory cells. ×670.

Schwann Cell Transplantation

Schwann cells are not normal occupants of the glial environment of the central nervous system. However, they are able to myelinate demyelinated central nervous system axons in situations where there is concurrent loss of both astrocytes and oligodendrocytes. This situation essentially leaves demyelinated axons outside the central nervous system and arises in certain disease states, or can be created by the injection of various gliotoxic chemical into white matter tracts. Such lesions are normally repaired largely by Schwann cells. If this normal repair re-

action is inhibited by local high-dose x-irradiation, the axons can be remyelinated by transplanted Schwann cells, which can be either placed over (Blakemore, 1977, 1980, 1984), or into the area of demyelination (Harrison, 1980; Duncan et al., 1981; Blakemore and Crang, 1985; Baron-Van Evercooren et al., 1991). Their subsequent behavior, *vis-à-vis* the demyelinated axons, parallels their behavior with neurites from cultured dorsal root ganglia so elegantly explored by the Bunges (Bunge and Bunge, 1983). Thus, they migrate along blood vessels and along the pial collagen and they require contact with collagen in order to myelinate (Blakemore and Crang, 1985).

Astrocytes deny Schwann cells access to demyelinated axons. Thus, when Schwann cells are injected into astrocyte-containing areas of demyelination (Duncan et al., 1981) or myelination failure, such as exists in the myelin-deficient rat, the extent of myelination achieved is very restricted (Duncan et al., 1988). When injected into normal tissue initial migration is followed by the disappearance of transplanted cells (Blakemore et al., 1986; Baron-Van Evercooren et al., 1992a). Transplanted Schwann cells have recently been demonstrated to migrate through normal tissue toward areas of demyelination (Baron-Van Evercooren et al., 1992b). Areas of demyelination have been repaired by transplanted Schwann cell lines (Baron-Van Evercooren et al., 1992b; Jung et al., 1993), and cross-species remyelination by Schwann cells has also been reported, namely, mouse/rat (unpublished observations); rat/mouse (Duncan et al., 1981; Baron-Van Evercooren et al., 1991) cat/rat (unpublished observations); rat/cat (Blakemore et al., 1987a). Schwann cell lines have been used in various transplantation studies, and, although such cells are capable of myelinating demyelinated axons, the ability to do this for some cell lines appears to decrease with increasing passage number (Jung et al., 1994).

Astrocytes

Studies on transplanted astrocytes differ from those primarily concerned with the behavior of oligodendrocyte lineage cells in two respects. First, the accurate description of the donor material that has been a feature of recent oligodendrocyte studies has not been matched in astrocyte studies because of our incomplete understanding of astrocyte development and the diversity of mature astrocyte forms. Second, the unavailability of astrocyte-deficient recipients equivalent to the myelination mutants used in oligodendrocyte studies means that studies on the behavior of transplanted astrocytes have mainly been carried out using normal recipients. This has necessitated the use of a wide variety of labels including species-specific antibodies (Jacque et al., 1986; Zhou et al., 1990), tritiated thymidine (Lindsay and Raisman, 1984; Emmett et al., 1991), microspheres (Emmett et al., 1988; Hatton et al., 1992), and lectins (Goldberg and Bernstein, 1987). In spite of the limitations imposed by both the labels used (see above) and the environment into which they are introduced, an important issue to emerge from these studies is the tendency for astrocytes to migrate away from the point of transplantation. This migration can be extensive within both neonatal (Suard et al., 1989; Zhou et al., 1990; Jacque et al., 1992) and adult central nervous system (Goldberg and Bernstein, 1987; Emmett et al., 1991), although a recent report indicates that the extent of migration decreases with increasing age of recipient (Zhou and Lund, 1993). Migrating astrocytes appear to take a variety of pathways during migration, of which the perivascular space is the most widely reported (Lindsay and Raisman, 1984; Goldberg and Bernstein, 1987; Emmett et al., 1988, 1991; Smith and Miller, 1991), although other routes may be used, such as in association with the glia limitans or ventricular wall (Goldberg and Bernstein, 1987; Hatton et al., 1992) or within fiber bundles (Zhou et al., 1990; Hatton et al., 1992; Jacque et al., 1992). Our own studies indicate that transplanted astrocytes migrating within the subpial space are able to enter the underlying white matter across an intact glia limitans (Franklin et al., 1993c). Currently, there is no clear consensus whether it is only immature astrocytes that migrate (Ignacio et al., 1990; Smith and Miller, 1991; Franklin and Blakemore, 1993) or whether mature astrocytes are also able to migrate (Emmett et al., 1991).

The factors that govern astrocyte migration appear to relate to the environment into which astrocytes are transplanted, rather than being an intrinsic property of the transplanted cells (Zhou et al., 1990; Jacque et al., 1991; Hatton et al., 1992; Zhou and Lund, 1993). This tendency for local environmental cues to override intrinsic determinants of behavior is also observed in the morphologies adopted by transplanted astrocytes when they become integrated into the host environment. Thus, transplanted astrocytes of striatal origin that migrate into the cerebellum will assume the morphology of cerebellar astrocytes rather than striatal astrocytes (Jacque et al., 1991).

The behavior of astrocytes within central nervous system lesions has been addressed using the x-irradiated ethidium bromide lesion. When purified populations of astrocytes are transplanted into a glia-

free environment consisting of demyelinated axons, the astrocytes are able to form an integrated astrocytic environment where the demyelinated axons are separated from one another by astrocyte processes (Franklin et al., 1993a) (Figure 58-8). This ability to integrate within glia-free lesions is disturbed by the presence of large numbers of Schwann cells (Franklin et al., 1992a) or meningeal cells (Franklin et al., 1992b). In these situations the astrocytes tend to form small, dissociated clumps of cells that exclude axons (Franklin et al., 1992a, 1992b). The ability of Schwann cells to disrupt the integrative behavior of astrocytes appears to be considerably reduced by the presence of cells of the O-2A lineage (see below). In certain circumstances, the introduction of certain astrocyte preparations into areas of demyelination is followed by extensive axonal degeneration, while, with other preparations, it is not (unpublished observations). The reason for these paradoxical results is at present unknown, but it likely reflects the heterogeneity of astrocytes, which will be difficult to explore until a fuller understanding of astrocyte diversity is available.

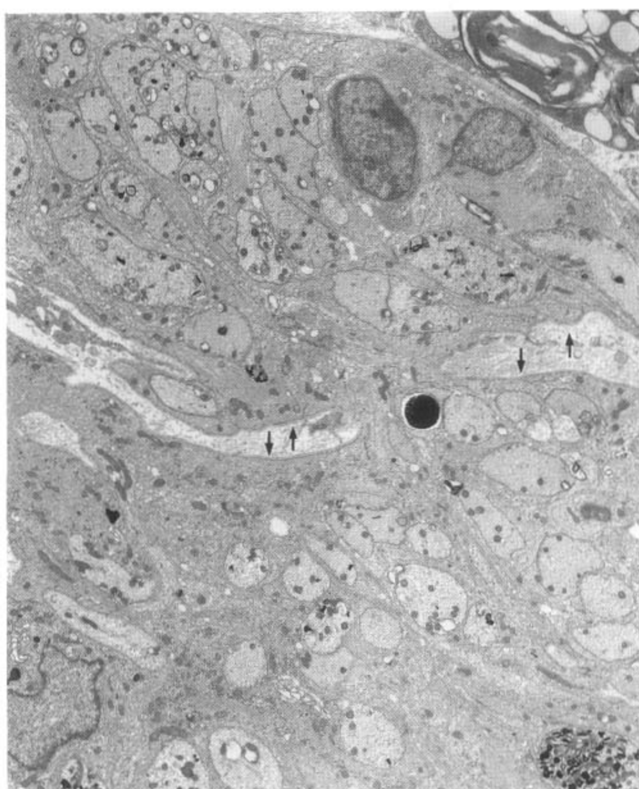


FIG. 58-8. The behavior of astrocytes within central nervous system lesions can be examined using the x-irradiated ethidium bromide lesion. Following transplantation of purified astrocyte cultures into demyelinating lesions, an integrated astrocytic environment around the demyelinated axons is formed with a border resembling a glia limitans (*arrows*). ×4000.

Transplantation of Progenitor Cells

Expansion of cells using growth factors or under the influence of conditionally acting oncogenes offers the prospect of obtaining large numbers of clonally derived cells for transplantation. The myelinating and repair potential of glial cell progenitors prepared using these techniques have been investigated in three studies. O-2A progenitors expanded *in vitro* by exposure to a combination of basic fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) for 3 weeks are capable of generating oligodendrocytes that myelinate demyelinated axons following transplantation (Crang et al., 1992; Groves et al., 1993) (Figure 58-9). However, if such cells are maintained on growth factors for many months prior to transplantation not all the cells dif-

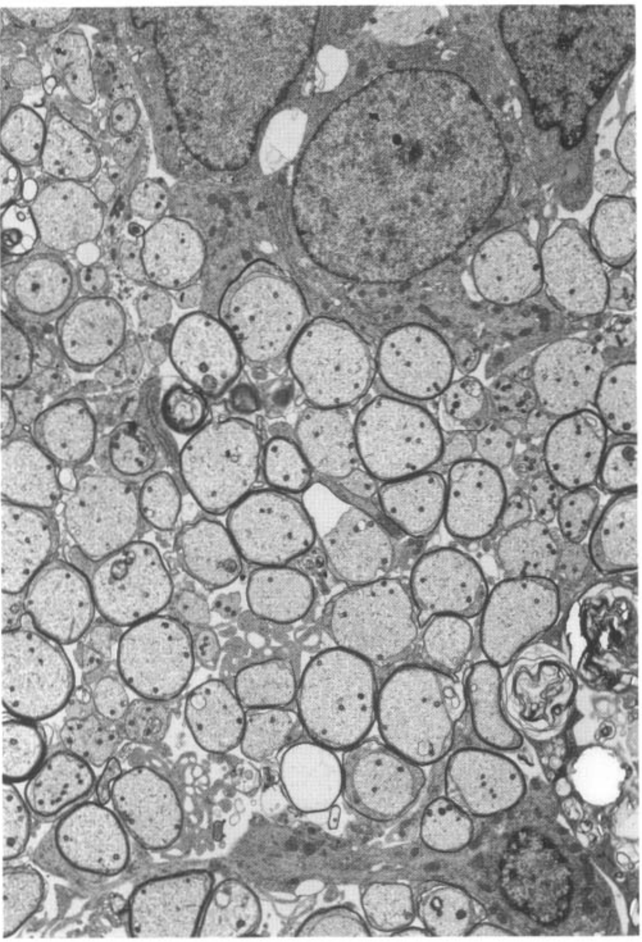


FIG. 58-9. The x-irradiated spinal cord ethidium bromide lesion provides a useful environment in which to examine the potential of glial progenitor cells to reconstruct a central nervous system glial environment. At 21 days following transplantation of O-2A progenitors expanded from optic nerve cultures by exposure to basic fibroblast growth factor and platelet-derived growth factor, extensive oligodendrocyte remyelination is found. ×4000.

ferentiate into oligodendrocytes on introduction into glial-free areas of demyelination (Groves et al., 1993). In this study the transplanted O-2A progenitors largely differentiated into oligodendrocytes. However, a different result was found following on transplantation of cloned progenitor cells produced using retroviral insertion of the temperature-sensitive SV40 large-T gene, since these cells gave rise to astrocytes as well as myelin-producing oligodendrocytes (Barnett et al., 1993; Trotter et al., 1993). These studies show that progenitor cells can be bipotential in pathological situations, a property that has not been demonstrated in normal development. An interesting difference was found between the two studies. In one study, the infection was made using mouse cells, and the resulting clonal cell lines contained both astrocytes (glial fibrillary acidic protein [GFAP+] cells) and progenitors on *in vitro* analysis prior to transplantation (Trotter et al., 1993), while in the other study the rat optic nerve-derived cell line was maintained in an undifferentiated state *in vitro* by continual exposure to PDGF and basic FGF (Barnett et al., 1993). On transplantation the mouse cell line gave rise to an integrated mixture of astrocytes and oligodendrocytes within the area of demyelination, while the rat cell lines gave areas that contained mainly one cell type or the other. In both cases not all cells differentiated and the ability of cells to differentiate into recognizable glial cells decreased with prolonged growth *in vitro*.

The Use of Glial Cell Transplantation to Study Cellular Interactions During Repair of Central Nervous System Demyelination

Demyelinated central nervous system axons can be remyelinated either by oligodendrocytes or Schwann cells. Which of these two types of repair occurs depends on the complex series of interactions between axons, cells of the oligodendrocyte and astrocyte lineages, and Schwann cells. Specific roles for these various cell types in determining the type of repair that follows demyelination have been revealed by creating situations where only certain cell types are present, or where the relative number of a particular cell type has been increased or decreased. This can be achieved by transplanting various glial cell cultures into x-irradiated and nonirradiated ethidium bromide lesions (Blakemore and Franklin, 1991).

The essential role of the astrocyte in creating environments where oligodendrocytes rather than Schwann cells can remyelinate central nervous system axons has been demonstrated using the nonirradiated ethidium bromide lesion. Following ethidium bromide injection into spinal cord white matter most of the axons are remyelinated by Schwann cells. *A priori*, one would predict that the balance of repair could be shifted toward oligodendrocyte remyelination by supplementing the number of oligodendrocytes by transplantation. However, when a central nervous system glial culture enriched for O-2A lineage cells is transplanted into the ethidium bromide lesion at the beginning of the repair process, the majority of axons are still remyelinated by host-derived Schwann cells (Blakemore and Crang, 1989). This observation indicates that when oligodendrocyte lineage cells are in direct competition with Schwann cells for demyelinated axons to remyelinate, then Schwann cell remyelination will prevail. The repair of the ethidium bromide lesion can, however, be changed in favor of oligodendrocyte remyelination by transplanting a mixed glial cell culture that contains a higher proportion of astrocytes (Blakemore and Crang, 1989). Moreover, the balance can also be shifted toward oligodendrocyte remyelination, albeit to a lesser degree, by transplantation of astrocytes alone (Franklin et al., 1991). These observations strongly support the concept that the presence of astrocytes favors oligodendrocyte remyelination, while their absence will favor Schwann cell remyelination. Astrocytes may also influence the "quality" of the myelin sheaths formed by oligodendrocytes (Franklin et al., 1993b).

The tendency for oligodendrocytes to be "outcompeted" by Schwann cell in the absence of astrocytes is only reversed when the former are present in sufficient numbers, and the Schwann cell contribution is limited. This situation can be created by using the x-irradiated ethidium bromide lesion, where, as has been discussed earlier, the host glial response is completely suppressed (Crang and Blakemore, 1989). In this situation, the cell types that interact with one another in the reconstruction of a glial environment are entirely governed by the composition of the transplant culture. When an oligodendrocyte lineage culture, depleted of astrocytes but containing the small contaminating population of Schwann cells invariably found in central nervous system cultures, is transplanted into an irradiated lesion most of the remyelination is by oligodendrocytes (Blakemore and Crang, 1989). This result also indicates that, while an astrocyte presence favors oligodendrocyte remyelination, it is not a necessity, a view that is supported by the ability of purified populations of O-2A progenitors to generate large numbers of myelinating oligodendrocytes in the absence of significant numbers of astrocytes (Groves et al., 1993). These studies indicate a dynamic balance between peripheral and central glia during the repair

of demyelinating lesions, where astrocytes are necessary to secure oligodendrocyte remyelination in the face of competition from Schwann cells.

This relationship between astrocytes and Schwann cells has been investigated in more depth by transplantation studies, revealing a further level of complexity. For it is now apparent that astrocytes are only able to restrict Schwann cell remyelination with the support of cells of the O-2A lineage. This conclusion was reached by the observation that, following transplantation of astrocyte cultures containing low numbers of Schwann cells and O-2A lineage cells into an x-irradiated lesion, extensive Schwann cell remyelination occurs (Crang and Blakemore, 1989; Franklin et al., 1992b). Thus, in spite of the presence of large numbers of astrocytes in the transplant, it is the small population of Schwann cells that expand to repair the lesion and not the small population of O-2A lineage cells. Indeed, in the absence of O-2A lineage cells, astrocytes may even enhance Schwann cell remyelination (Franklin et al., 1992b). The paradoxical behavior of astrocytes with regard to Schwann cell remyelination can be related to the arrangements adopted by transplanted astrocytes in the presence or absence of O-2A lineage cells. When O-2A lineage cells and astrocytes are present, together they combine to create large areas of reconstructed central nervous system white matter. In contrast, in the absence of O-2A lineage cells, astrocytes form dissociated clumps of cells from which many axons are excluded and hence are available for remyelination by Schwann cells (Franklin et al., 1992b, 1993b). These results suggest a role for O-2A lineage cells in influencing the ability of transplanted astrocytes to integrate within central nervous system lesions, an essential behavior if axons are not to be myelinated by Schwann cells. Thus by using the two types of ethidium bromide lesion and cultures of varying composition, it has been possible to demonstrate that the cell defined in tissue culture as the type-1 astrocyte is capable of excluding Schwann cell from the central nervous system (Blakemore and Crang, 1989). However, for this cell to fulfil this function in pathological situations, cells of the O-2A lineage are also required (Franklin et al., 1992a).

The recognition that O-2A lineage cells are essential for promoting astrocyte control of Schwann cell invasion into glial free lesions, together with the demonstration that mouse oligodendrocytes will myelinate rat axons (Crang and Blakemore, 1991) has formed the basis for the creation of a central nervous system environment, which resembles the chronically demyelinated central nervous system plaque of multiple sclerosis and provides a new model of immune-mediated demyelination (Blakemore, 1992). In this system, a chimeric cell suspension of isogeneic rat astrocytes and xenogeneic mouse O-2A lineage cells is transplanted into a demyelinating ethidium bromide lesion made in normal rat spinal cord, the survival of the mouse component of the transplant being controlled by immunosuppression. Thus, in immunosuppressed recipients, the mouse cells survive and remyelinate the demyelinated axons, and also assist in the establishment of the transplanted rat astrocytes, so that the lesion is remyelinated with central rather than peripheral myelin. If the immunosuppression is subsequently removed, the mouse cells are rejected and the axons are demyelinated for a second time. In contrast to the nontransplanted ethidium bromide lesion (Graça and Blakemore, 1986), the axons are remyelinated by oligodendrocytes. This is because a glial limitans exists, created by the transplanted rat astrocytes. Using this system it is possible to examine remyelination of an area of immune-mediated demyelination, which is self-limiting and not directed primarily against oligodendrocyte specific epitopes, as occurs in other models of allergic encephalomyelitis.

A modification of this system creates a further model. If at the same time as initiating rejection of the mouse cells by removing immunosuppression, the lesion area is exposed to 40 Gy of x-irradiation, the host-mediated remyelination that follows rejection of the mouse myelination can be suppressed. As a result, the demyelinated axons are left in an astrocyte environment which models the chronically demyelinated multiple sclerosis plaque (Blakemore and Crang, 1994).

CONCLUSION

Glial cell transplantation is a new and rapidly developing area of glial cell biology. It is becoming clear that the technique provides the investigator with a degree of experimental control over *in vivo* situations that has hitherto been available only for *in vitro* studies. This has enabled studies to be undertaken in developing or pathological central nervous system to validate concepts of glial cell behavior developed in tissue culture and to address fundamental questions about central nervous system regeneration. Already a clearer picture is emerging of the complex cell-cell interactions that occur in pathological situations in the central nervous system. The value of glial cell transplantation as an experimental procedure has also opened up the exciting prospect of using this technique to alter or augment

glial cell populations in clinical situations in order to stimulate or modify regeneration in the central nervous system.

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59 Microglia in experimental neuropathology

JOCHEN GEHRMANN AND GEORG W. KREUTZBERG

Dedicated to Henry deF. Webster

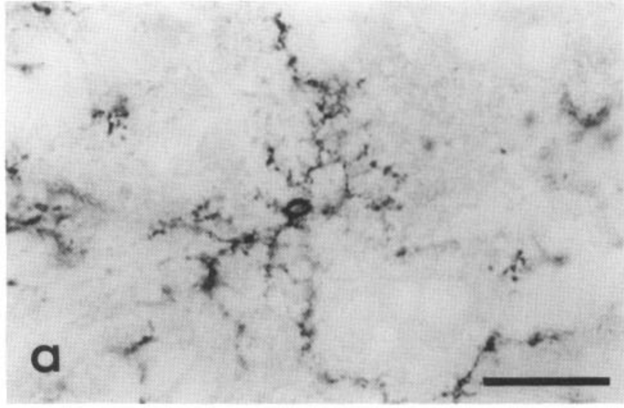
Microglia are ubiquitously distributed in nonoverlapping territories throughout the nervous system. These resident cells contribute up to 20% of the total glial cell population in the brain (Perry and Gordon, 1988; Lawson et al., 1990; Peters et al., 1991). Microglia can be best identified by means of silver impregnations, histochemical stainings (Kreutzberg and Barron, 1978), lectin stainings (Streit and Kreutzberg, 1987), and by several monoclonal antibodies that recognize antigens specific for cells of the monocyte/macrophage lineage (Dijkstra et al., 1985; Perry et al., 1985; Perry and Gordon, 1988; Gehrmann and Kreutzberg, 1991; Flaris et al., 1993). At the light microscopic level, all these stains show resting microglia to possess a characteristic ramified morphology with several crenellated processes. Their morphology appears to depend to some extent on their location (Perry et al., 1985; Streit et al., 1988; Gehrmann and Kreutzberg, 1991) (Figures 59-1A,B). At the ultrastructural level, resting microglia have a characteristic nucleus with a prominent heterochromatin but a rather scanty cytoplasm (for review see Peters et al., 1991 and Chapter 10) (Figure 59-1C).

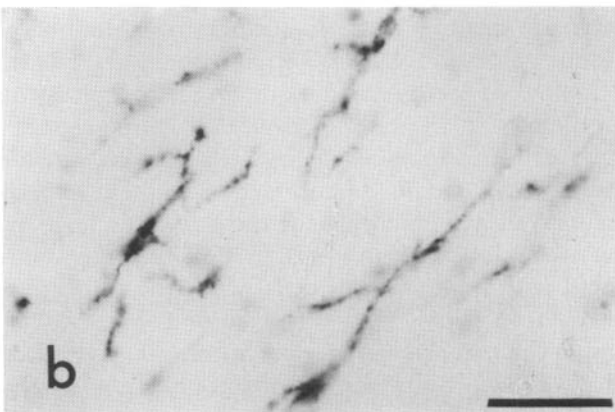
An important but still controversial issue of microglial biology concerns the cells' origin. In line with del Rio Hortega's initial postulate (Rio Hortega, 1919, 1932), the generally held view is that the resident microglia are of mesodermal origin, possibly derived from a bone marrow or embryonic precursor cell. These cells and/or lineage-related peripheral monocytes/macrophages invade the central nervous system at an early stage via the vasculature and eventually give rise to the typical process-bearing resident microglia (Ling and Wong, 1993). In contrast, a microglial progenitor cell has been thought to develop from nutrionally deprived astroglial cultures (Richardson et al., 1993), thus raising again the question of a neuroectodermal origin of microglia.

The close antigenic and lineal relationship of microglia to monocytes/macrophages, however, is further underlined by the fact that all monoclonal antibodies raised so far against microglia also cross-react with macrophages in peripheral tissues (Dijkstra et al., 1985; Perry et al., 1985; Gehrmann and Kreutzberg, 1991; Flaris et al., 1993). In this respect, it is interesting to note that microglia display a characteristic channel pattern distinct from peripheral monocytes/macrophages (Kettenmann et al., 1990).

Linked to the question of microglial origin is the problem of microglial turnover, that is, the replacement of intrinsic microglia by cells residing outside the brain. Based on bone marrow chimera experiments, resident microglia in the adult rat have an extremely low (i.e., lower than 1%) turnover compared with bone marrow cells, while the percentage of replaced cells is high in the leptomeninges (60%) and in the perivascular compartment (30%) (Hickey et al., 1992). In this respect, macrophages/microglia in a perivascular position are particularly important for brain immune surveillance at a site close to the blood-brain barrier. Two types of cells can be distinguished. First, are the perivascular cells, which are distinct from the smooth muscle-related lineage of pericytes. These perivascular cells express macrophage markers, are ultrastructurally enclosed within the basal lamina, can be induced to phagocytose and constitutively express major histocompatibility complex (MHC) class II antigens (Graeber et al., 1989, 1992). Second, are the perivascular microglia, which form a subpopulation of resident, parenchymal microglia. These perivascular microglia are found within the central nervous system parenchyma proper, but make direct contact with the tissue side of the adjoining basal lamina, thus forming part of the perivascular glia limitans similar to perivascular astrocytic endfeet (Gehrmann and Kreutzberg, 1991; Lassmann et al., 1991). In an immunopathological context, they rapidly express MHC class II antigens and furthermore have the capacity to phagocytose (Vass and Lassmann, 1990; Lassmann et al., 1991; Gehrmann et al., 1993a).

While the function of resting microglia is still largely unknown, it has become clear from observations in neuropathology that resting microglia rapidly undergo characteristic morphological





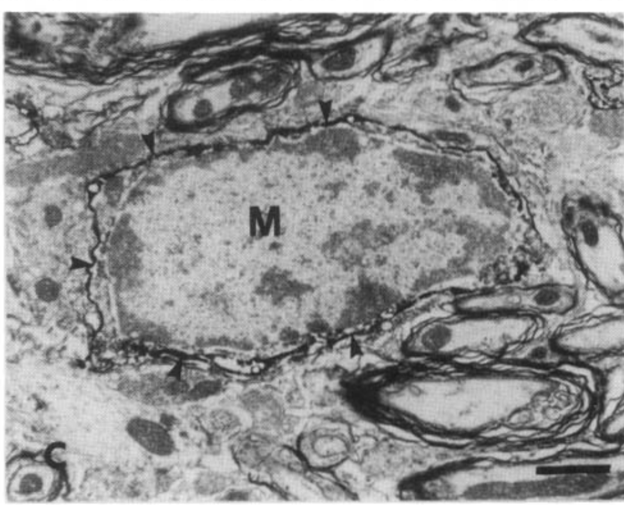


FIG. 59-1. Morphology of resting microglia in the rat. MUC 102 immunocytochemistry. While microglia (A) in the gray matter have a stellate morphology, (B) they display a bipolar morphology in the white matter. (C) At the electron microscopic level an immunostained microglia (*M*) (the *arrowheads* indicate the sites of the diaminobenzidine (DAB) reaction product) has a typical nucleus with a prominent heterochromatin and a rather scanty cytoplasm. Scale bar (Figures A and B) = 25 μm; Figure C = 1 μm.

changes in response to injury. It was Franz Nissl who first clearly recognized the capacity of intrinsic microglia to transform rapidly from a resting to an activated state in response to injury (Nissl, 1899; 1904). The terminology used to describe activated microglia has been confusing. These cells have been termed, for example, inflammation globules (*Entzündungskugeln*), foamy cells (*Schaumzellen*), granule cells (*Körnerzellen*), lattice cells (*Gitterzellen*), or rod cells (*Stäbchenzellen*). With the advent of monoclonal antibodies in recent years it has become possible to study more precisely the process of microglial activation. Microglial activation appears to follow a rather stereotypic pattern of cellular responses and occurs in a graded fashion. Activation becomes apparent mainly in the form of proliferation (in contrast to astrocytes which tend not to proliferate, but become hypertrophic) (Cammermeyer, 1965; Kreutzberg, 1968; Sjöstrand, 1965; Graeber et al., 1988), recruitment to the site of injury, morphological and immunophenotypical changes, as well as alterations in their functional properties, for example, the release of cytokines (Frei et al., 1987; Streit et al., 1988; Giulian et al., 1989; Gehrmann et al., 1992a, 1992d; Finsen et al., 1993; Flaris et al., 1993).

In terms of morphological changes, many transitional and intermediate morphologies of activated microglia occur, a fact which might have led to the confusing terminology of microglia in the past. In the first step of activation, microglia usually become hypertrophic with several stout processes but do not become phagocytic. If neuronal and/or terminal degeneration occur in the brain parenchyma, activated microglia further transform into phagocytic cells (Streit and Kreutzberg, 1988; Gehrmann et al., 1991a) and, under certain conditions morphologically resemble round, lipid-laden cells with a morphology similar to that of foamy macrophages (Brierley and Brown, 1982).

In this respect, the model of sublethal and lethal nerve injury has been particularly useful, since the blood-brain barrier remains unimpaired, allowing one to study exclusively the response of intrinsic microglia. All these studies underline the capacity of resident microglia to transform rapidly into intrinsic phagocytes of the brain, a process, which, due to its pronounced cytotoxic potential, seems to be under strict control *in vivo*. In addition to resting microglia, two types of activated microglia can be distinguished: an activated, but nonphagocytic microglia and an activated, phagocytic cell, which is equivalent to an intrinsic brain phagocyte. The most conspicuous immunophenotypical changes include the increased or even *de novo* expression of surface

molecules, some of which, such as MHC class I and II antigens, are normally found only on cells of the immune system. In line with their capacity to phagocytose and to release several immunomodulatory substances, these immunophenotypical properties indicate that microglia in fact form part of an intrinsic immune mechanism of the nervous system. Upon even remote lesions and/or pathological conditions lacking obvious neuropathological changes microglia appear to be rapidly activated as part of an early central nervous system immune defense system.

THE MICROGLIAL RESPONSE TO EXPERIMENTAL BRAIN INJURY

Peripheral Nerve Injury

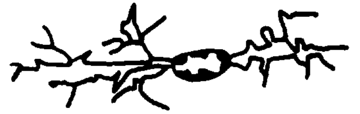
Peripheral nerve injury leads to well-defined retrograde and/or anterograde changes in the central nervous projection areas. Unlike most other experimental models, these remote nerve lesions (the brain itself is not touched) have the advantage of leaving the blood-brain barrier intact. Thus the reaction of intrinsic microglia can be studied in the complete absence of any infiltrating hematogenous macrophage. In particular, the facial nerve axotomy paradigm has proved to be suitable for studying the graded response of intrinsic microglia to injury. The facial nerve is cut unilaterally at its exit at the foramen stylomastoideum, and the remote microglial response is then studied in the facial nucleus of origin with the nonoperated side serving as an internal control. Following facial nerve axotomy a defined microglial reaction occurs (Figures 59-2 to 6). Within 3 to 4 days after the axotomy resident microglia, but not astrocytes, proliferate mainly around the injured facial motoneurons (Cammermeyer, 1965; Kreutzberg, 1966; Graeber et al., 1988a) (Figure 59-3). From 24 hours onward, several marker molecules, such as the CR3 complement receptor (Graeber et al., 1988b), are increased on hypertrophic, but still ramified, microglia. Activated microglia further express the intermediate filament protein, vimentin (Graeber et al., 1988c), the ectoenzyme 5′-nucleotidase (Kreutzberg and Barron, 1978; Schoen et al., 1992) (Figure 59-4), leukocyte function associated antigen-1 (Moneta et al., 1993), the CD4 antigen and MHC class I, and with some delay also class II molecules (Streit et al., 1989a, 1989b). Interestingly, the increased expression of MHC class II molecules appears to be under corticoid control, since treatment with dexamethasone downregulates its expression (Kiefer and Kreutzberg, 1992). Following facial nerve axotomy, activated microglia furthermore show an early de novo expression of the amyloid precursor protein (APP). Since APP immunoreactivity is found ultrastructurally within the endoplasmic reticulum of activated microglia, and since cultured microglia also synthesize APP mRNA, microglia appear to contribute to some extent to APP formation *in vivo* or even amyloidogenesis itself (Banati et al., 1993a). In addition, activated microglia show an increased expression of receptors for granulocyte-macrophage and macrophage colony-stimulating factors, both of which may regulate microglial proliferation (Raivich et al., 1991). Another cytokine, which is induced following facial nerve transection and most likely localized on activated microglia, is the transforming growth factor-beta 1 (TGFβ1) (Kiefer et al., 1992). From day 4 after axotomy onward activated microglia ensheath the soma of the injured facial motoneurons with their broadened cellular processes. As seen in semithin sections, one facial motoneuron is then usually covered by three to four microglia. Ultrastructurally, activated perineuronal microglia begin to displace afferent synaptic terminals from the motoneuron surface (Figure 59-6). This process, first described by Blinzinger and Kreutzberg (1968), is now generally referred to as synaptic stripping. It might lead to a synaptic deafferentation at somatic and stem dendritic sites, which in turn are important for the synaptic reorganization following neuronal injury.

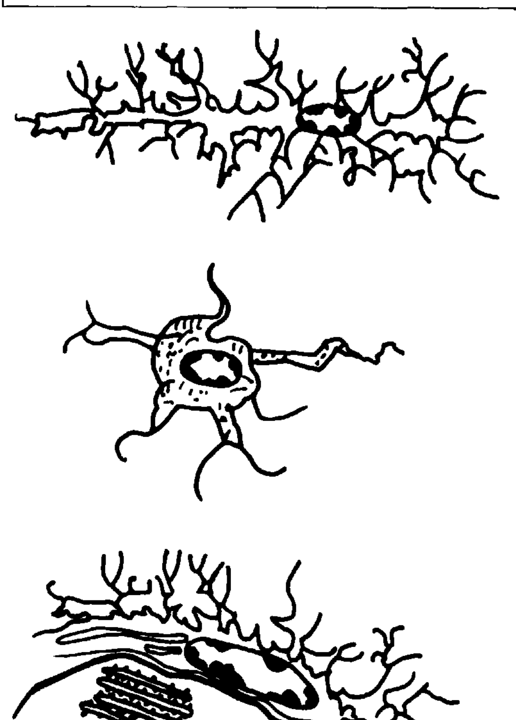
Under all these conditions, microglia become activated but do not become phagocytic. However, if facial motoneuron death is induced experimentally by the injection of toxic ricin into the facial nerve, microglia rapidly become phagocytic (Streit and Kreutzberg, 1988) (Figure 59-2). They transform into true brain macrophages, which eventually remove the neuronal debris. These experimental observations following sublethal (caused by facial nerve axotomy) and lethal (caused by ricin injection) motoneuron injury underline the fact that the microglial response to injury occurs in a graded fashion. First, microglia become activated, but not phagocytic, then, in a second step, if neuronal and/or terminal degeneration occur, they transform further into intrinsic phagocytes of the brain (Streit et al., 1988; Gehrmann et al., 1991a).

In contrast to facial nerve axotomy, which leads to a pure retrograde neuronal response, axotomy of the sciatic nerve induces a variety of neuronal and axonal reactions in different locations (Gehrmann et al., 1991b). While retrograde reactions occur in the ventral gray matter of the lumbar spinal cord and the corresponding dorsal root ganglia, transgan-

RESTING MICROGLIA

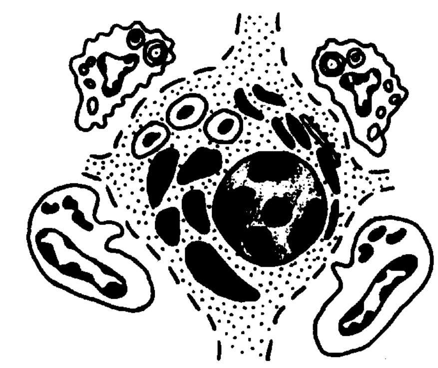
In their resting state microglia have a typical ramified morphology which is adapted to their microenvironment (i.e. in the white matter they have a bipolar morphology, and in the grey matter a stellate morphology). In addition microglia have a characteristic nuclear morphology with a prominent heterochromatin. They express several molecules constitutively (i.e. the CR3 complement receptor, the MUC 101 & 102 epitopes, LFA-1, α-D-Galactose containing glycoconjugates which are recognized by GSI-B_4) while other molecules are only expressed at low levels (CD4, vimentin, MHC class I and II molecules) or are absent from resting microglia (ED 1-3, Ki-M2R).





ACTIVATED, BUT NON-PHAGOCYTIC MICROGLIA

In response to sublethal neuronal injury (i.e. after facial nerve transection) resting microglia proliferate (for comparison see Fig. 3) and undergo characteristic morphological changes mainly by becoming hypertrophic. Proliferating microglia in a perineuronal position are found in direct apposition to the surface of the injured motoneuron whereby apparently detaching afferent synaptic terminals from the neuronal surface ("synaptic stripping"). These activated microglia, however, do not become phagocytic. In addition, they show a markedly increased expression of several marker molecules (CR3, MUC 101 & 102, GSI-B4, LFA-1, CD4, vimentin, MHC class I and II antigens and APP). They also appear to newly synthesize TGF-β1.



ACTIVATED, PHAGOCYTIC MICROGLIA

Neuronophagia: in response to lethal neuronal injury (i.e. after toxic Ricin injection into the facial nerve) microglia become activated and furthermore triggered to transform into phagocytic cells. The marker molecule expression is similar to that observed after sublethal injury with the exception that under certain conditions these cells aquire typical "macrophage" markers such as ED1.

FIG. 59-2. Graded response of microglia to neuronal injury. Resting microglia have either a bipolar or a stellate morphology and express several marker molecules (characteristic of cells of the monocyte/macrophage lineage) constitutively. Each cell is furthermore characterized by a typical nuclear morphology with a prominent heterochromatin. Following peripheral nerve transection, microglia change into an activated, but nonphagocytic state. They show an increased expression of several marker molecules and undergo characteristic morphological changes mainly by becoming hypertrophic. At the ultrastructural level activated microglia are found in a close perineuronal position detaching synaptic terminals from the neuronal surface. Under conditions of neuronal cell death, activated microglia further transform into phagocytic cells containing typical phagosomes. [Adapted from Streit et al. (1988), with permission.]

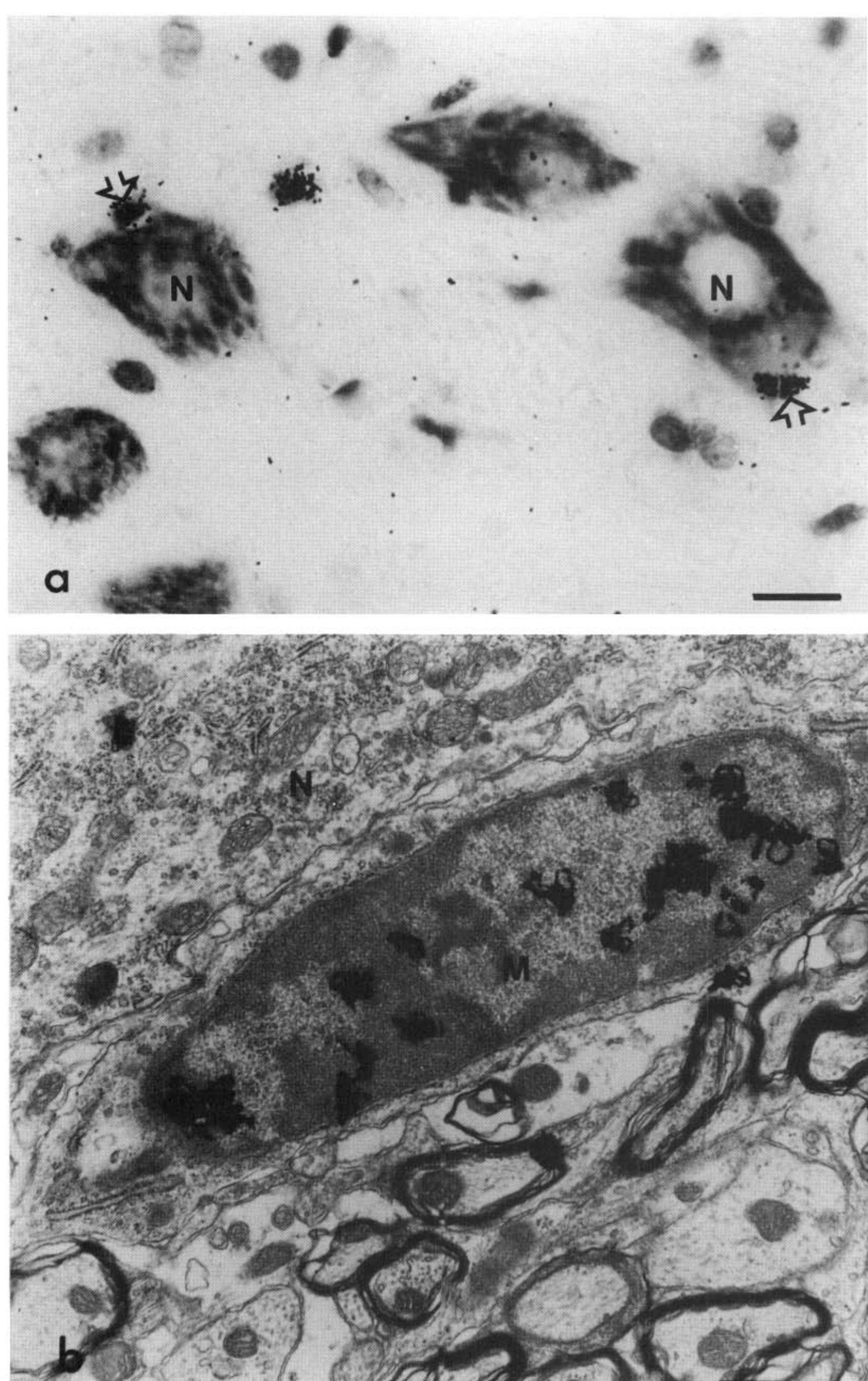


FIG. 59-3. Microglial proliferation in the rat facial nucleus 4 days after facial nerve transection. (3H)Thymidine autoradiography. (**A**) At the light microscopic level, several small glial cells are labeled. (**B**) Electron microscopy demonstrates that the silver grains are localized on the nucleus of a perineuronal microglia (*M*). Scale bars: Figure A = 25 μm, Figure B = 1 μm (Photographs by courtesy of Manuel B. Graeber.)

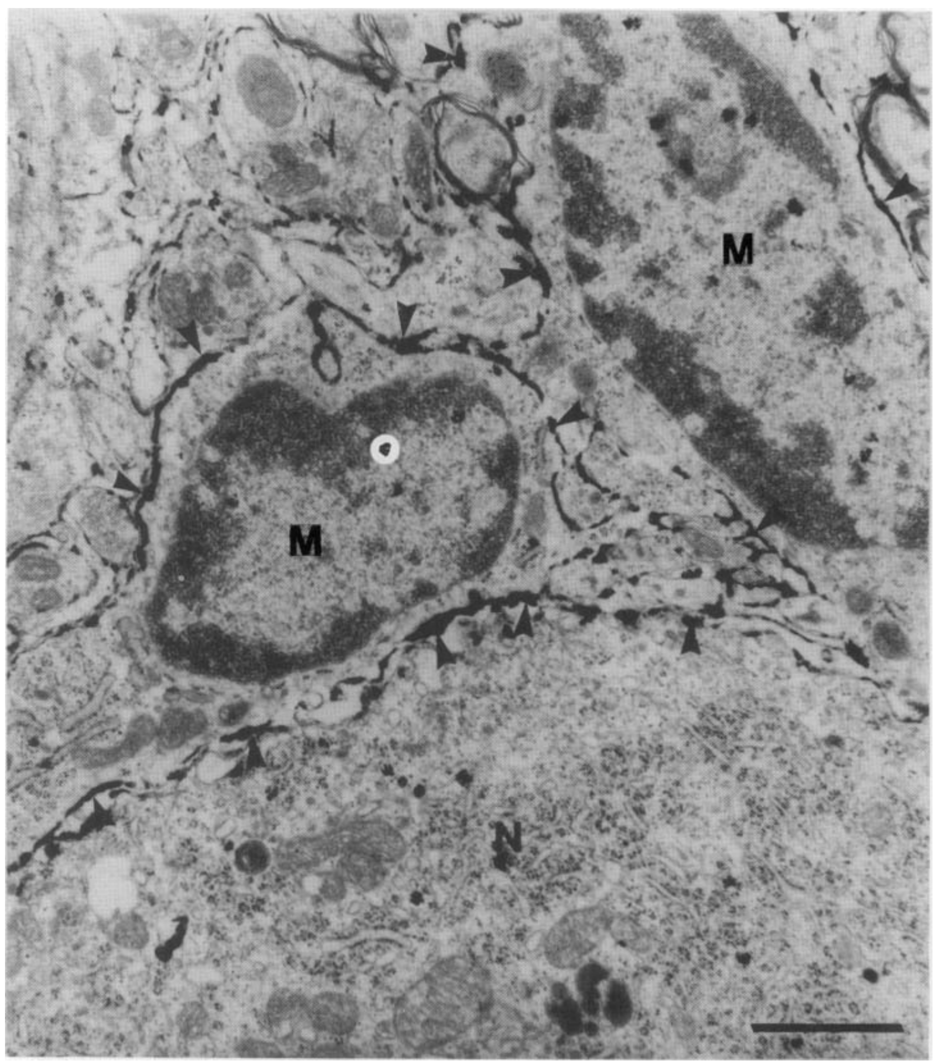


FIG. 59-4. 5′-Nucleotidase histochemistry in the rat facial nucleus 7 days after facial nerve transection. At the electron microscopic level the surface of two perineuronal microglia (*M*) is strongly labeled by 5′-nucleotidase reaction product (*arrowheads*). *N*, facial motoneuron. Scale bar = 1 μm.

glionic changes take place in the dorsal gray matter and the ipsilateral nucleus gracilis due to the degeneration of the central branches of the primary afferent sensory fibers and terminals. Microglial reaction and proliferation are observed from 24 hours after axotomy onward at all these locations (Gehrmann et al., 1991b), but the rapid and remote microglial response in the nucleus gracilis is especially intriguing (Gehrmann et al., 1991b). In this area, early molecular changes in axonal and synaptic terminals that precede the appearance of argyrophilic degeneration products seem to be sufficient to induce microglial proliferation and activation. This early reaction further supports the concept that resident microglia are central nervous system cells which respond to even subtle and remote pathological stimuli.

Wallerian Degeneration

Transection of the optic nerve, usually performed behind the globe 1.0 to 1.5 cm distant from the contralateral superior colliculus, leads to an anterograde Wallerian degeneration, which develops along the optic tract and in the contralateral superior colliculus. Apart from the directly injured optic nerve stump, there is no evidence for a gross impairment of blood-brain barrier function in these areas. Within the denervated optic nerve the rapid and gradually extending reaction of microglia/macro-

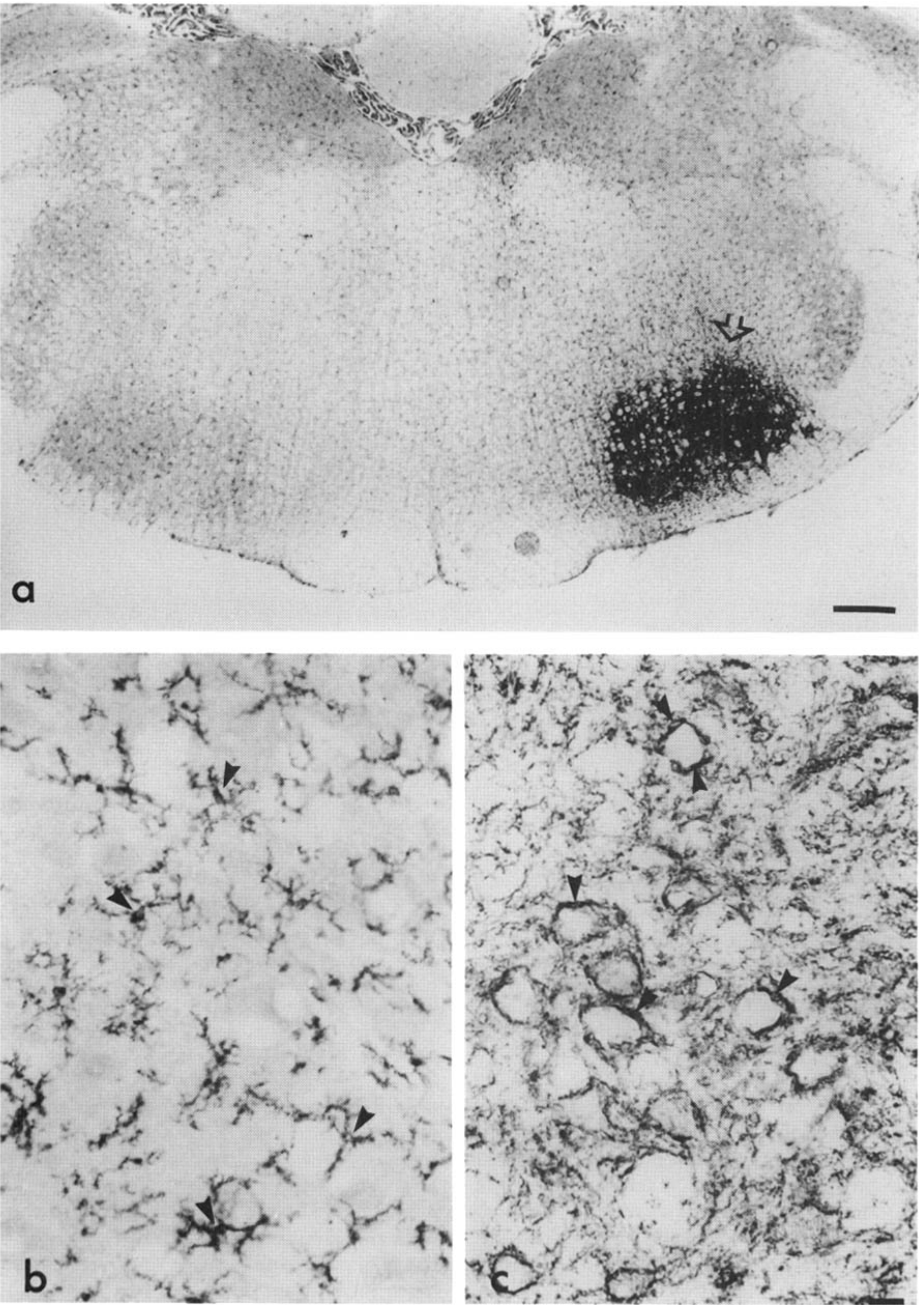


FIG. 59-5. Response of microglia to transection of the rat facial nerve. MUC 102 immunocytochemistry. (A) The overview of a rat brainstem shows that the microglial immunoreactivity is strongly increased on the facial nucleus (*arrow*) ipsilateral to the side of facial nerve transection after 5 days. Scale bar = 500 μm. [From Gehrmann and Kreutzberg (1991) with permission.] (B) In the intact control nucleus microglia have a ramified morphology characteristic of resting cells. (C) Five days after facial nerve transection the facial motoneurons are surrounded by strongly immunoreactive perineuronal microglia. Scale bar = 25 μm.

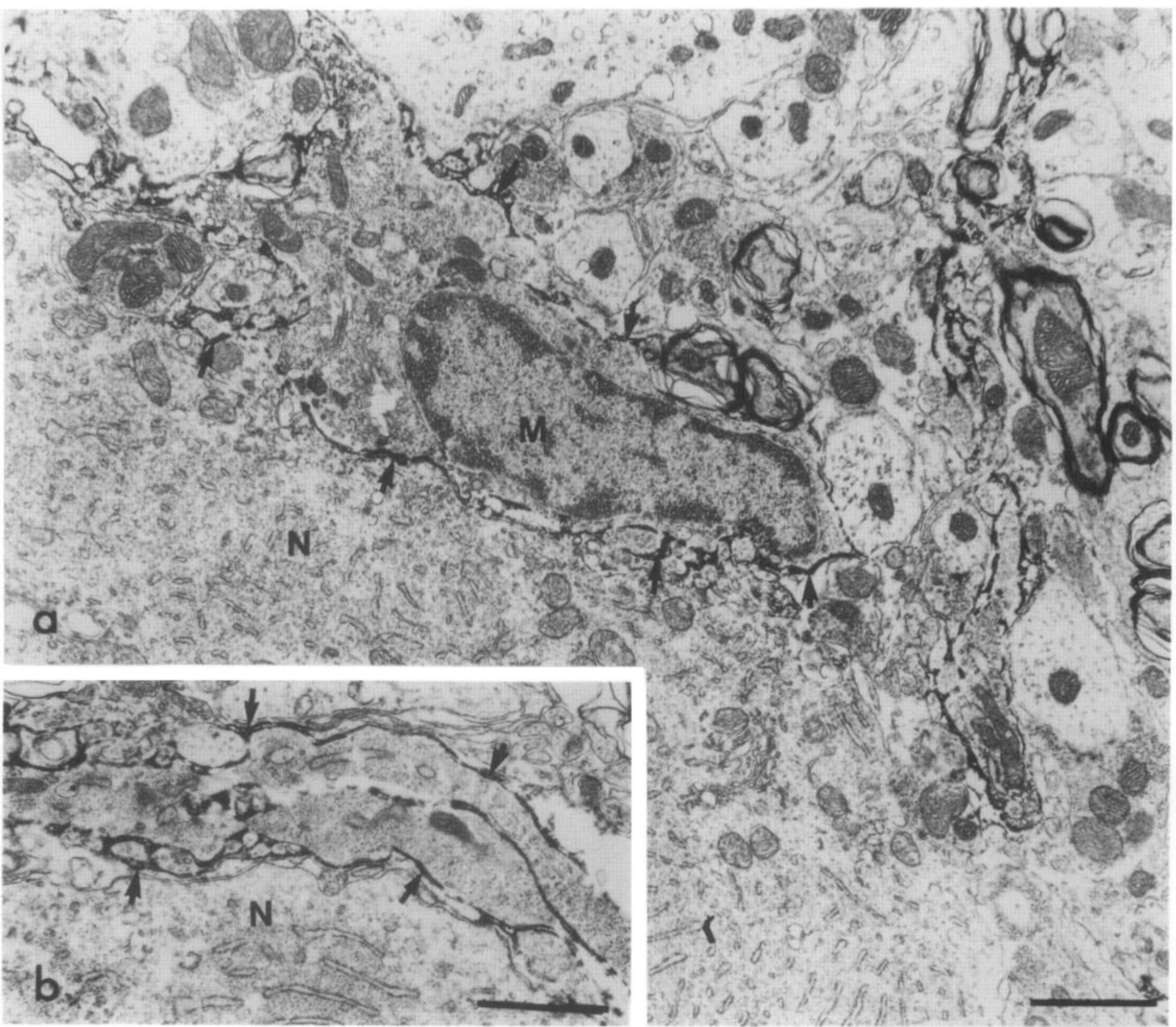


FIG. 59-6. Ultrastructure of a perineuronal microglia in the operated facial nucleus. (**A**) MUC 102 immunoelectron microscopy (*arrowheads* indicate the sites of the diaminobenzidine (DAB) reaction product). Five days after facial nerve transection, a perineuronal activated microglia (*M*) is situated in the close vicinity of an injured facial motoneuron (*N*). Higher magnification in Insert **B** shows stacks of microglial processes which make direct contact with the neuronal surface. Scale bars = 1 μm. [From Gehrmann and Kreutzberg (1991) with permission.]

phages is an early feature of Wallerian nerve degeneration (Konno et al., 1989; Rao and Lund, 1989). These cells usually have a morphology similar to that of foamy macrophages, which is compatible with the notion that they are involved in the removal of the slowly degenerating myelin sheath. In addition, macrophages obtained from transected optic nerves produce a favorable substrate for nerve regeneration *in vitro,* suggesting that the macrophage response (similar to the situation after peripheral nerve axotomy) might also enhance central nervous system repair mechanisms (David et al., 1990). In the superior colliculus, a spatially distinct reaction of microglia occurs. Whereas increased expression of the CR3 complement receptor, of the leukocyte common antigen and of MHC class I molecules is observed on activated microglia throughout the deafferented colliculus, *de novo* expression of CD4 and MHC class II molecules is restricted to the stratum opticum (Flaris et al., 1993). Both types of reactions reach a peak 7 days after optic nerve transection. Since the stratum opticum is the nerve cell layer to which the myelinated axons of the retinal ganglion cells project, this restricted expression of class II molecules support the assumption that myelin and/or myelin breakdown products could be instrumental in regulating class II expression on microglia (Smetanka et al., 1990).

Anterograde Degeneration in the Deafferented Dentate Gyrus

Lesion of the entorhinal cortex leads to the degeneration of its projection to the outer molecular layer of the dentate gyrus where the corresponding granule cells are depleted of their afferent input. Following a short period of lesion-induced axonal and terminal degeneration, reinnervation of the molecular layer takes place due to the sprouting of cholinergic fibers arising from the septal nuclei. Activated microglia, but not reactive astrocytes, appear as early as 24 hours postlesion in the deafferented dentate gyrus (Fagan and Gage, 1990; Gehrmann et al., 1991a). The microglial reaction peaks around day 3 postlesion and is no longer easily detected after 8 days. It is thus terminated before the onset of collateral sprouting events. Ultrastructurally, dark terminal degeneration is detectable within 24 hours postlesion (Gehrmann et al., 1991a). At the same time, activated microglia begin to phagocytose these degeneration products. Therefore, terminal degeneration most likely provides the stimulus for microglial proliferation, activation, and transformation into intrinsic phagocytes, which remove the degenerating material. Interestingly, the early and transient microglial reaction precedes the later and persisting astrocyte reaction. Although the involvement of cytokines and trophic factors is still controversial in this lesion paradigm, the sequential activation of microglia and astrocytes could have functional importance. Activated microglia might induce astrogliosis, while the ensuing astrocytic reaction might subsequently support regenerative sprouting events (Fagan and Gage, 1990).

Penetrating Brain Injury

The most commonly studied model of direct (penetrating) brain injury is the stab wound model in which a penetrating injury to the cortex is performed stereotactically to a depth of approximately 1 mm from the cortical surface. The ensuing cellular response is mainly limited to the site of the needle tract and the tissue directly surrounding the lesion. It resembles a wound healing reaction, and due to the obvious blood-brain barrier damage, involves both intrinsic microglia as well as hematogenous macrophages in addition to other infiltrating blood cells such as leukocytes, granulocytes, and monocytes (Giulian et al., 1989). Round hematogenous macrophages are mainly found within the lesion itself, whereas process-bearing, hypertrophic intrinsic microglia occur at the edge of the lesion. Both types of mononuclear phagocytes, intrinsic and extrinsic, are detectable in increased numbers within the first 10 hours postlesion. Their reactivities peak around day 2 and decline until 10 days postlesion (Giulian et al., 1989). At day 5 postlesion ramified hypertrophic microglia localized at the edge of the injury notably express MHC class II and the CD4 antigen while faintly expressing the ED1 marker (Flaris et al., 1993). In general, the microglial reaction following direct penetrating trauma to the rat brain is less pronounced than that observed, for example, in response to an inflammatory lesion. Interestingly, at day 2 (i.e., at the peak of mononuclear phagocytic activity) considerable amounts of IL-1 can be identified in the lesion area (Giulian et al., 1989). Since IL-1 appears to control astrogliosis (Giulian and Lachman, 1985; Giulian et al., 1986), it has been postulated from these results that IL-1 released by activated macrophages/microglia could be responsible for the sequential activation of astrocytes starting from day 5 onward. Other mediator substances which could be involved are interleukin-6 which has been measured by microchamber dialysis techniques (Woodroofe et al., 1991) and, also TGFβ1 which could be localized on macrophages/microglia at the edge of a stab wound by *in situ* hybridization (Lindholm et al., 1992).

Ischemia

Transient arrest of the cerebral blood circulation leads to neuronal cell death in selectively vulnerable brain regions, such as the dorsolateral striatum and the hippocampal CA1 and CA4 regions (Spielmeyer, 1925; Vogt, 1925; Scholz, 1953; Pulsinelli, 1982). Unlike neurons, glial cells survive such a transient ischemic injury. It is now well established that glial cells further respond to ischemia by becoming activated. The resulting neuropathological process has been defined as that of a selective neuronal necrosis. While astrocytic reactions, as indicated by increases in glial fibrillary acidic protein immunoreactivity, are well documented (Schmidt-Kastner et al., 1990; Petito et al., 1990), relatively little is known about the reactions of resident microglia except for some information pertaining to their role as phagocytes in hypoxia (Brierley and Brown, 1982).

Based on studies on transient forebrain ischemia induced by four-vessel occlusion, the microglial reaction has been examined more intensively (Morioka et al., 1991; Gehrmann et al., 1992a, 1992c; Finsen et al., 1993). Microglia appear to be activated as early as 20 minutes following the ischemic injury (Morioka et al., 1991). From day 1 onward, they proliferate and show an increased expression of sev-

eral surface molecules, most conspicuously MHC class I and II molecules. Although there is some discrepancy in the time course of the microglial reaction (most likely due to different time intervals of the ischemic injury). A pronounced microglial reaction occurs in the dorsolateral striatum and the hippocampal CA1 (with an accentuation of the stratum pyramidale and lacunosum moleculare) and CA4 (Figure 59-7). In the stratum pyramidale of the CA1 sector, microglia have been observed at the ultrastructural level to be phagocytosing degenerated material which resembles that of the foamy macrophages described in infarcts (Gehrmann et al., 1992c) (Figure 59-7). Immunoelectron microscopy indicates that perivascular microglia are also actively involved in the phagocytosis process and are rapidly inducible for MHC class II expression (Gehrmann et al., 1992c). Interestingly, resident microglia appear to make frequently contact with neighboring structures, such as the neuronal soma or dendrites (even while those are still intact) as if they were scanning their microenvironment for possible tissue damage (Gehrmann et al., 1992c). This again suggests that microglia function as a network of resident cells which can become rapidly activated in case of tissue damage.

In the stratum radiatum of the CA1 sector, activated microglia assume a typical rod cell morphology and are ultrastructurally observed to phagocytose the degenerating dendrites of the CA1 pyramidal neurons. In contrast, there is almost no microglial reaction in the hippocampal CA3 which is relatively spared by the ischemic injury in most models of ischemic injury.

In addition, a delayed microglial reaction can be observed in other locations with a less pronounced selective vulnerability such as the substantia nigra and the anterior parts of the thalamus (Gehrmann et al., 1992b). In white matter tracts hypoxia/ischemia leads to diffuse axonal swelling and injury (Hirano et al., 1967), which is accompanied by a markedly increased MHC class II expression on microglia (Gehrmann et al., 1992b). In ischemia, as well as in other models of experimental injury, the microglial reaction furthermore serves as an early and sensitive marker for the temporal and spatial distribution pattern of impending neuronal damage. In the neocortex, for example, activated microglia are either concentrated in layer 3 (probably reflecting the selective vulnerability of layer 3 neurons) or arranged in columns to the cortical surface. This arrangement in columns most likely reflects both anterograde and retrograde changes induced in cortical neurons by the simultaneous neuronal cell death in interconnected thalamic nuclei (Gehrmann et al., 1992b). In addition, activated microglia in ischemia have recently been shown to express newly the mRNA of TGFβ1 and IL-1 (Wießner et al., 1993).

What causes the rapid microglial reaction in ischemia? Manifest neuronal cell death is certainly a strong stimulus for the activation of microglia which become phagocytic and remove the tissue debris. Since full-blown neuronal cell death lags behind the early activation of microglia, manifest cell death does not appear to be the only candidate. In addition to early ultrastructural and biochemical neuronal changes, other factors such as release of exitotoxic amino acids, cytokines, and also increased ion fluxes could play a role. Since ischemia is associated with pronounced changes in extracellular potassium, and since microglia are sensitive to changes in potassium due to their unique potassium channel pattern (Kettenmann et al., 1990), we have postulated that potassium might be among the factors that trigger the activation of microglia in ischemia. Following cortical spreading depression, which leads to increased potassium fluxes across membranes, microglia become transiently activated throughout the cortex in the absence of neuropathic changes (Gehrmann et al., 1993b).

While it is clear that the microglia will respond to neuronal damage by becoming activated, it is still possible that the early microglial reaction further augments tissue damage due to the pronounced cytotoxic potential of this cell. Interestingly, treatment with drugs that supposedly interfere with macrophage reactions, such as chloroquine, improve the neurological status in animals with spinal cord ischemia (Giulian and Robertson, 1990). In addition, the microglial reaction is almost absent in rats treated with the *N*-methyl-D-aspartate (NMDA) antagonist MK-801 (Streit et al., 1992), although it has been argued that MK-801 acts primarily by rescuing neurons and thereby abolishes the most crucial stimulus for microglial activation. Obviously, the question of microglial cytotoxicity and other functions of this cell type in ischemia deserve further attention, since interference with the process of microglial activation could provide new clues to the treatment of neurological diseases such as stroke.

Experimental Autoimmune Encephalomyelitis

The nervous system has long been considered an immunologically privileged site. This view has been based on four main arguments: (1) lack of lymphatic drainage, (2) seclusion from the periphery by a tight blood-brain barrier, (3) impaired rejection of transplants, and (4) lack of intrinsic antigen-presenting

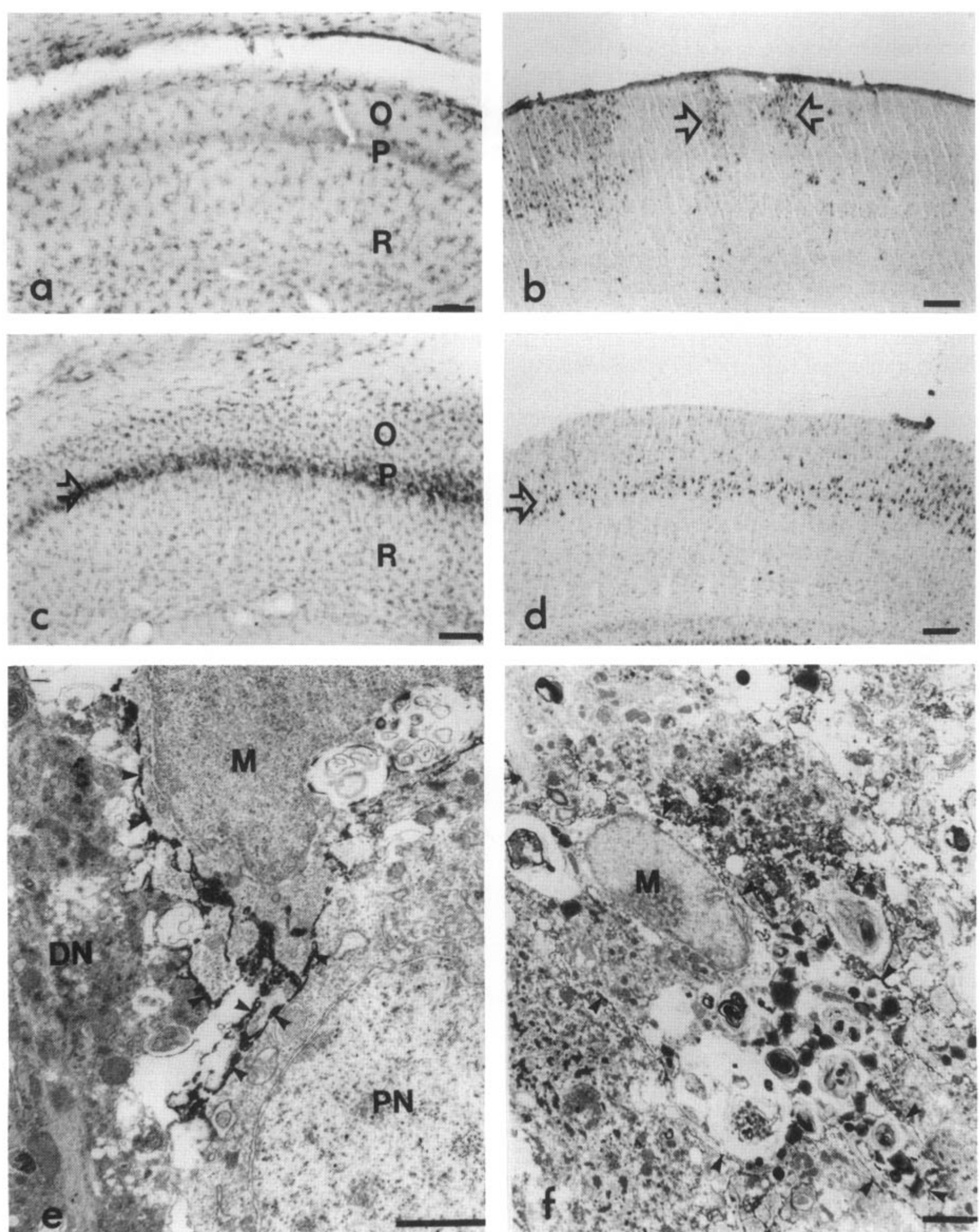


FIG. 59-7. Microglial response to global ischemia (induced by 30 minutes of a four-vessel occlusion) in the hippocampal CA1 sector. (**A,B,C,D**) Light microscopy. Three days after the ischemic injury (*C*) activated microglia are prominent throughout all layers of CA1, but particularly in the stratum pyramidale (*P*) O, str. oriens; *P*, str. pyramidale; *R*, str. radiatum. In the parietal cortex MHC class II immunoreactive microglia are arranged either in columns to the cortical surface (*B*) or are concentrated within layer 3 (*D*). (**E,F**) MUC 102 immunoelectron microscopy (The *arrowheads* indicate the sites of the diaminobenzidine (DAB) reaction product). (*E*) An immunoreactive microglia is interposed between two types of CA1 neurons (i.e., a pale type of neuron (*PN*), that may eventually survive, and a dark, degenerating neuron (*DN*). (*F*) Example of phagocytic microglia (with a morphology similar to that of a "foamy macrophage") which contains several phagocytosis products within its enlargened cytoplasm. Scale bars: Figures A–D = 25 μm; E and F = 1 μm. [Figures B and D from Gehrmann et al. (1992b), Figures E and F from Gehrmann et al. (1992c), with permission.]

cells (for review see Wekerle et al., 1986). Although these properties render the nervous system an immunologically distinct organ, the immune system obviously locates foreign antigens within the nervous system and subsequently induces immune reactions (e.g., inflammation) therein. One way by which immune surveillance is performed in the nervous system has been elucidated by Wekerle et al., (1986). Immunocompetent, activated T lymphocytes can enter the brain in an apparently random fashion subsequently patrolling the brain in search of their specific antigen (Hickey et al., 1991).

The histopathological hallmark of T cell-induced autoimmune diseases are perivascular lesions, consisting mainly of T lymphocytes, B lymphocytes, and macrophages. Within the inflammatory lesion many of the mononuclear cells are macrophages, predominantly ED1-positive, a few ED3-positive, but almost no involvement of ED2-positive cells (Flaris et al., 1992). As demonstrated first by Vass et al. (1986) and by Matsumoto et al. (1986), there is furthermore a generalized reaction of resident microglia within the parenchyma: Microglia proliferate, show an increased expression of several surface molecules, most conspicuously MHC class II antigens, and transform morphologically into either a rod-shaped or round, ameboid form. In the vicinity of the perivascular cuff, hypertrophic reactive astrocytes encase the primary core of the inflammatory lesion (consisting mainly of mononuclear cells) and are thus arranged in a way as if to prevent the interaction between infiltrating activated T lymphocytes and activated microglia (Matsumoto et al., 1992).

The functional role of this macrophage/microglial reaction is not yet fully understood. Both types of cells could initiate and maintain the immune reaction by virtue of their *in vitro* antigen-presenting capacity. In this respect, two types of resident central nervous system cells could be important. First, perivascular ED2-positive cells (Graeber et al., 1989), which have also been demonstrated in human brain (Graeber et al., 1992), could be involved. These cells express MHC class II molecules constitutively and can be triggered to phagocytose foreign material. Second, perivascular microglia, which are intraparenchymal cells but also form part of the glia limitans, could also be involved. (Gehrmann and Kreutzberg, 1991; Lassmann et al., 1991) (Figure 59-8). These cells can also be rapidly induced to phagocytose as well as to express MHC class II antigens. Immunoelectron microscopy has indeed shown an activation of these perivascular microglia in experimental autoimmune encephalomyelitis (EAE) (Gehrmann et al., 1993a).

In addition, macrophages/microglia could be involved in mediating tissue damage both by phagocytosis and the release of harmful mediators. In fact, depletion of macrophages prior to the onset of disease by injection of either silica or liposomes attenuates the disease (Huitingta et al., 1990). Bone marrow chimera experiments by Hickey et al. (1992) have demonstrated that the turnover of resident microglia is rather low in the adult central nervous system, whereas a high percentage of perivascular and meningeal cells is repopulated from the bone marrow. Interestingly, EAE can be equally well induced in bone-marrow chimeras as well as in sham controls (Lassmann et al., 1993). This indicates a more pronounced role of perivascular and meningeal macrophages in initiating central nervous system autoimmune disease than of resident microglia.

Brain Abscess

The brain responds to a bacterial infection by the formation of a typical bacterial abscess surrounded by a fibrous tissue capsule. A brain abscess can be experimentally induced by injection of *Staphylococcus aureus*-loaded agarose beads into the cortex (Flaris and Hickey, 1992). In the early phase of infection, there is a widespread infiltration of neutrophils, granulocytes, and monocytes/macrophages into the necrotic area surrounded by an edema. In the second phase of disease, usually referred to as chronic cerebritis (i.e., between 4 and 8 days postinoculation) ED1-, ED3-, but also strongly ED2-positive macrophages along with some lymphocytes infiltrate the edema zone around the necrotic core. This influx, particularly the appearance of ED2-positive cells, takes place just before collagen deposition and capsule formation occur from day 9 onward (Flaris et al., 1993). Activated microglia external to the capsule scar then start to appear along with a concomitant astroglial reaction. Since the immunological reactions in brain abscess are distinct from those observed in T cell-mediated EAE (the latter resembling a T cell-mediated reaction of the delayed type hypersensitivity type), this model might provide an interesting approach for studying microglial reactions to acute infections.

Experimental Autoimmune Neuritis

The peripheral nervous system counterpart of EAE is experimental autoimmune neuritis (EAN). Either sensitization of susceptible animals to peripheral nervous system tissue (Waksman and Adams, 1955) or the passive transfer of autoaggressive T line cells specific for the P2 or the P0 protein of the peripheral

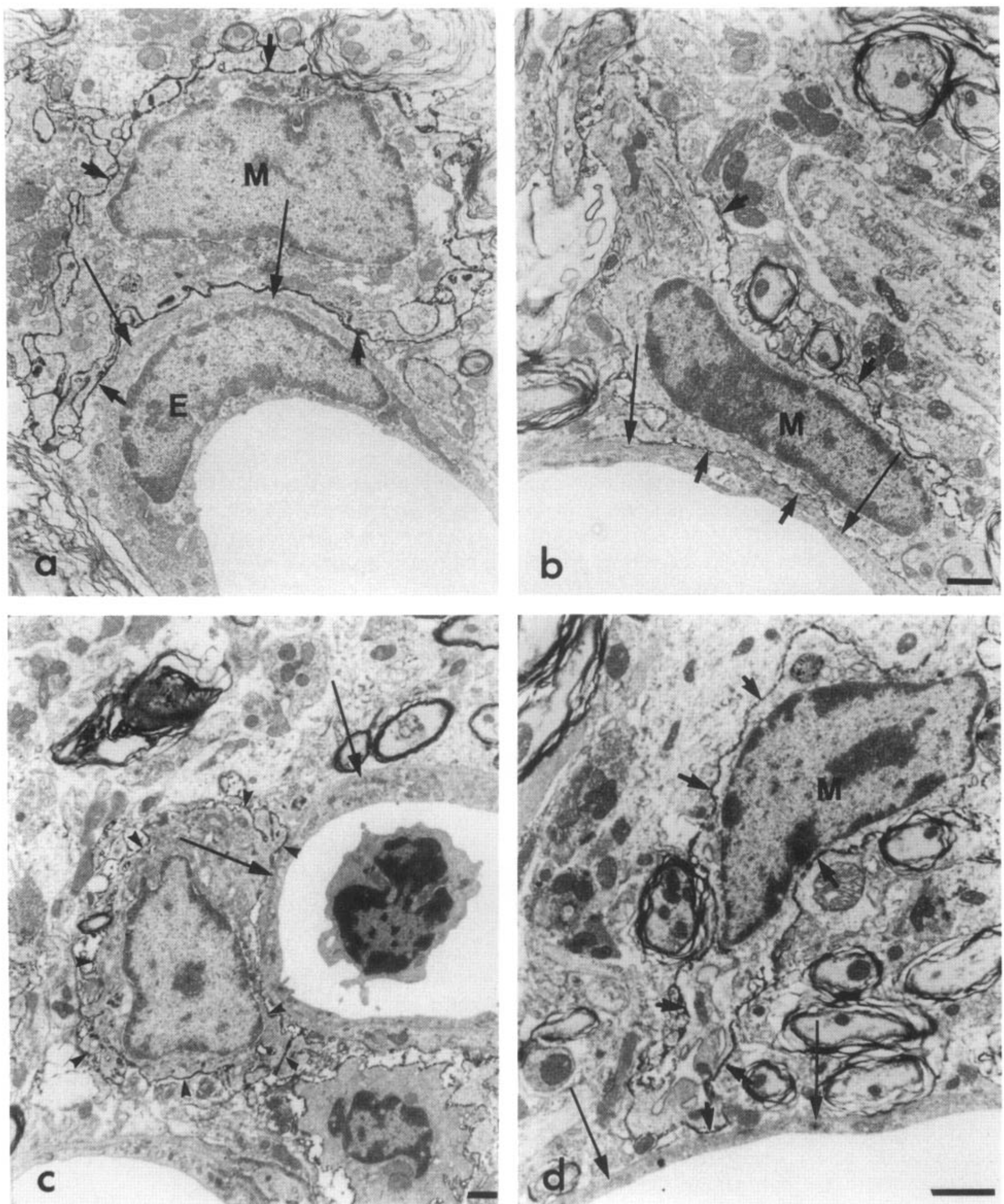


FIG. 59-8. Ultrastructure of perivascular microglia in experimental autoimmune encephalomyelitis (EAE) (*A* and *C*) and autoimmune neuritis (EAN) (*B* and *D;* 7 days after T cell transfer). (*A, B*): OX-6 immunoreactivity (indicating the presence of MHC class II molecules). (*C, D*) MUC 102 immunoreactivity. The arrowheads indicate the sites of the reaction product. (**A**) At an early stage of disease development in EAE (i.e., 4 days after T cell transfer), a perivascular microglia expresses high levels of MHC class II molecules on its surface (cf. Figure B). In addition, it extends several cellular processes which extend its surface area. (**B**) In contrast, in EAN a perivascular microglia expresses rather low levels of MHC class II molecules on its surface (cf. Figure A). (**C**) At the peak of clinical disease (i.e., after 7 days) numerous immunoreactive perivascular microglia and/or invading macrophages (due to impaired blood-brain barrier function) are found in the vicinity of blood vessels. (**D**) An immunostained perivascular microglia is directly incorporated within the perivascular glia limitans. Scale bars = 1 μm. [Figure A: from Gehrmann et al. (1993a), Figures B and D from Gehrmann et al. (1992d), with permission.]

nervous system myelin sheath leads to a pure peripheral nervous system autoimmune disease (Linington et al., 1984). Related to this autoimmune peripheral nervous system disease, a pronounced microglial reaction occurs within the spinal cord at sites that are topographically related to the main areas of peripheral nervous system inflammation (Gehrmann et al., 1992d, 1993a). This microglial response is most conspicuous in the ventral and dorsal gray matter of the lumbar, thoracic and cervical spinal cord. However, it is more pronounced in the lumbar and thoracic spinal cord than in the cervical spinal cord, since peripheral nervous system damage is more severe in the sciatic nerve and the lumbosacral fibers than in the brachial nerve and the cervical nerve roots. Interestingly, at least in the model of passive transfer EAN, this microglial reaction occurs very rapidly, preceding even to some extent the pathological changes in the peripheral nerve, such as inflammation and edema formation. Microglial activation in this process becomes apparent by proliferation, immunophenotypical changes, most conspicuously an increased expression of MHC class II molecules, morphological changes, and the release of cytokines such as TGFβ 1 (Kiefer et al., 1993). Similar to the situation after peripheral nerve transection, activated microglia are found to surround motoneurones in the ventral gray matter. Ultrastructurally, these perineuronal microglia are found to displace afferent synaptic terminals from the neuronal surface (Figure 59-9). In addition to the spinal cord, a microglial reaction also takes place in remote projection areas within the lower brainstem. These areas include the nucleus gracilis (the projection field of the lumbar primary afferent sensory fibres) and the nucleus cuneatus (the projection field of the cervical fibers). This microglial reaction is strictly confined to the terminal projection fields themselves and is not seen in the neighboring tissue. It is correlated to some extent with the presence of degenerating axonal and synaptic terminals, which are then gradually removed by phagocytic microglia. The rapid activation of microglia in EAN at sites remote from the primary peripheral nervous system lesion suggests that rapid remote signaling might occur in autoimmune inflammation.

Neurotoxin Lesions

Direct injection of neurotoxins into the brain results in neuronal injury and, furthermore, induces a vigorous inflammatory reaction. Unlike remote brain lesions, such as the facial nerve axotomy paradigm, these toxic lesions massively disrupt the blood-brain barrier, thus allowing the influx of hematogenous cells into the lesioned area. Intracerebral injection of kainate or ibotenate leads to a massive macrophage/microglial reaction at the site of injection while in sham-operated controls (given an intracerebral saline injection) only a reaction along the needle tract is found similar to that observed following a stab wound (Coffey et al., 1990; Andersson et al., 1991; Finsen et al., 1993). Within the lesioned area a high percentage of these reactive cells appears to be blood-derived based on three observations: (1) the blood-brain barrier is massively impaired; (2) the reactive cells share many morphological and immunophenotypical features with peripheral monocytes/macrophages and (3) systemic irradiation prior to the injury diminishes the response which is in line with the assumption that blood-derived monocytes are recruited in large numbers from the blood into the lesioned area (Coffey et al., 1990). Excitotoxic amino acids have been particularly implicated in the pathogenesis of neurological disorders. While commonly used excitotoxic amino acids such as kainate and ibotenate lead to rather nonselective forms of neuronal injury, injection of quinolinic acid into the rat striatum leads to a selective necrosis of GABAergic neurons. In addition to a strong macrophage/microglial reaction in the injured striatum itself, microglial activation occurs in a temporarilly and spatially organized pattern also in striatal projection areas such as the globus pallidus and the substantia nigra (Toepper et al., 1993). Moreover, a microglial response takes place in areas (e.g., the ventrolateral thalamus) that are only indirectly connected to the lesioned striatum. While anterograde degeneration of striatal projection neurons most likely causes the microglial reaction in first-order projection areas, other mechanisms, possibly neuronal hyperexcitation following removal of inhibitory striatal input, could be responsible for the microglial response in remote, only indirectly linked projection areas.

Experimental Gliomas

Microglia exhibit a pronounced cytotoxicity toward tumor cells *in vitro* (Frei et al., 1987). Macrophage/microglial reactions occur in and around human brain tumors as originally described by Penfield (1924). Implantation of rat glioma cell lines, such as the RG-2 glioma cell line, into the cerebrum offer an experimental approach to study the microglial response to brain tumors (Morioka et al., 1992). Depending to some extent on the number of injected cells and their growth kinetics, massive brain tumors

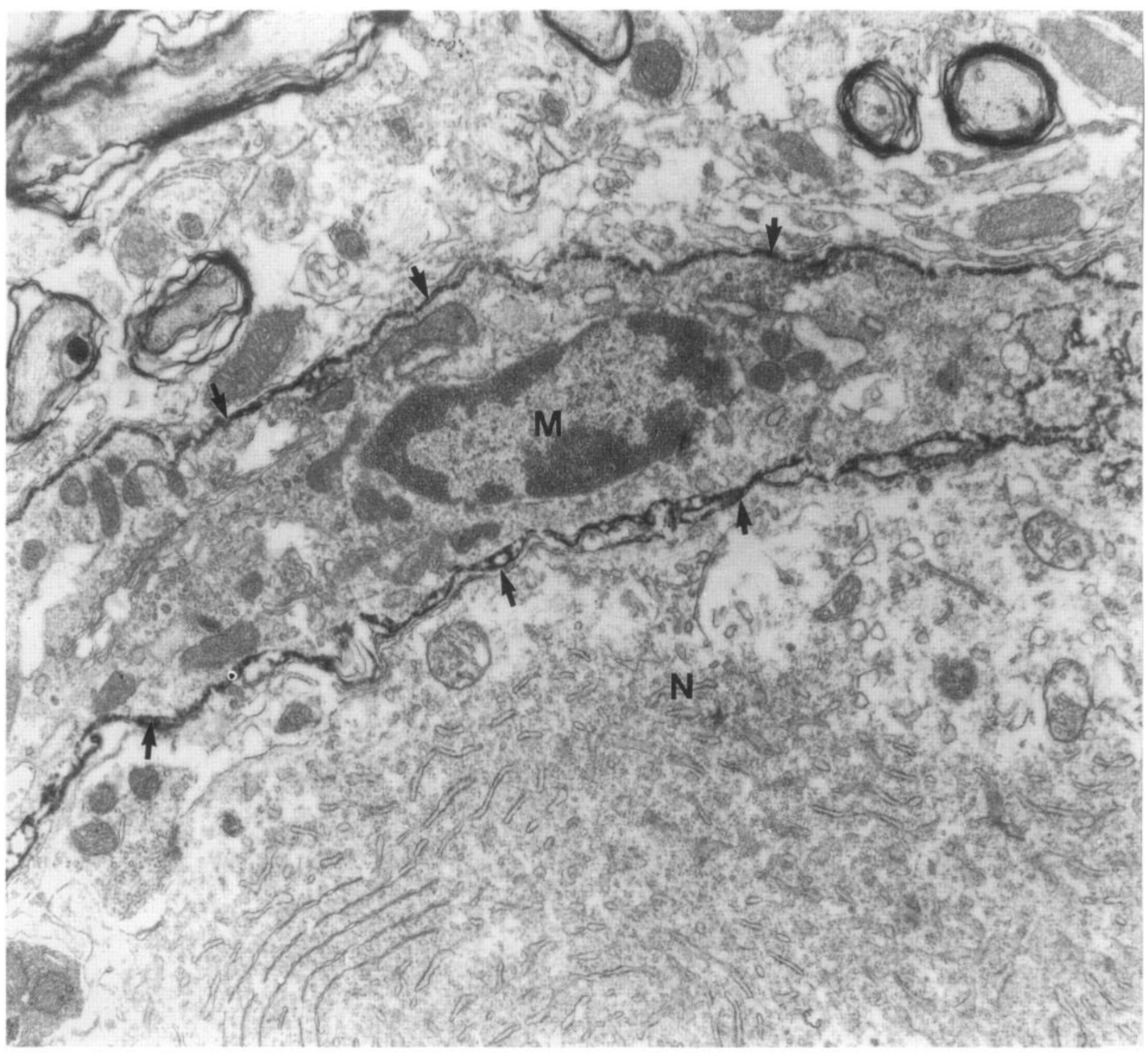


FIG. 59-9. Ultrastructure of a perineuronal microglia in experimental autoimmune neuritis. MUC 102 immunoelectron microscopy (*arrowheads* indicate the sites of the reaction product). Microglia is found in the ultimate vicinity of a motoneuron surface in the ventral gray matter of the lumbar spinal cord 7 days after T cell transfer. Scale bars = 1 μm. [From Gehrmann et al. (1992d), with permission.]

develop often with central necrosis and bleeding. The tumor mass is surrounded by a dense band of reactive cells, which sometimes extends into the contralateral hemisphere via the corpus callosum. From the center to the periphery of the tumor a gradient of reactive cells occurs. Within the center most cells morphologically and immunophenotypically resemble blood-derived macrophages, while toward the periphery the percentage of these cells diminishes in favor of intrinsic microglia-derived brain macrophages (Morioka et al., 1992). Although the tumor is thus heavily infiltrated by potentially cytotoxic cells, there is hardly any evidence for a pronounced *in vivo* tumor cytotoxicity of macrophages/microglia. Whether this occurs as a result of a suppressive activity of the tumor cells on microglia or as a result of impaired function of microglia, is one of the key questions in understanding their contribution to tumor immunology.

Transplantation

Interest in transplantation immunology has increased due to experimental clinical trials with intracerebral grafting of fetal human dopaminergic

brain tissue to patients with Parkinson's disease. Intracerebral rejection of xenogenic grafts is impaired in the brain compared to grafts placed at an extracerebral site, an observation that has supported the view of the brain as an immunologically privileged site (Medawar, 1948). Nevertheless, histoincompatible xenogenic central nervous system grafts are prone to undergo rejection (Finsen et al., 1991). In contrast, graft rejection is less pronounced and occurs with a delay in allogenic transplants. Due to their pronounced immunological properties, microglia seem to be particularly involved in graft rejection and phagocytosis. Studies on neural mouse xenografts into adult rat recipient brains have demonstrated that within 3 weeks following transplantation the graft gets infiltrated with leukocytes, T lymphocytes, and, most strikingly, macrophages. After 5 weeks only a heavily infiltrated residual graft remains (Finsen et al., 1991). Due to impaired blood-brain barrier function (as evidenced by massive leakage of serum proteins into the graft) the majority of the latter cells is probably blood-derived. In the host brain surrounding the graft resident microglia become activated, and exhibit strikingly increased MHC class I and class II antigen expression. In contrast, astrocytes express little class I immunoreactivity, and no class II. They rather respond by increased glial fibrillary acidic protein immunoreactivity (Finsen et al., 1991) and do not phagocytose except for terminals (Raisman and Field, 1990). Host macrophages and activated host as well as donor microglia might act as immunostimulatory cells on CD4-positive T-helper cells. These CD4-positive T cells are essential for the rejection of central nervous system grafts as shown by lymphocyte depletion studies. They most probably become stimulated by host and/or donor class II-positive macrophages/microglia. In contrast, the increased class I expression on donor glial cells (including microglia and astrocytes) makes these cells susceptible to cell lysis by host-derived CD8-positive T-cytotoxic cells. Interference with this crucial macrophage/microglial response might thus lead to improved graft survival in the brain.

Viral Infections

Microglia may respond to viral infections in two principal ways: they either become activated but not primarily infected, or they are the primary targets of a virus. The former mechanism takes place in the case of herpes simplex virus (HSV) infection, whereas a microglial viral tropism has been observed both experimentally and clinically in retroviral infections. Infection of rats with HSV type I by corneal scarification leads to a pronounced neuronal infection first via cranial nerves, mainly the trigeminal nerve (Weinstein et al., 1990). One week postinoculation (i.e., at the onset of severe clinical disease) the infection has spread to neurons throughout the brain, including the entire brainstem, thalamus, hypothalamus, cerebellum, and cortical layers 1 to 3. At the sites of infection the tissue is heavily infiltrated with granulocytes, T lymphocytes, and monocytes. In addition, microglia become activated as indicated mainly by dramatically increased MHC class I and II expression. While this microglial reaction is initially confined to the primary sites of infection, at the peak of infection (i.e., by days 8 to 10) activated microglia are detectable throughout the entire brain, including areas that are apparently free of obvious infection (Weinstein et al., 1990). In HSV-1 infection, a generalized microglial activation thus occurs, and activated class II-positive microglia may well serve to present viral antigen to CD4-positive T lymphocytes thereby augmenting the immune attack to the brain.

In contrast, murine retroviruses, such as the recombinant wild mouse ectotropic retrovirus (FrCasE), show a primary microglial tropism with both resident microglia and perivascular cells becoming heavily infected (Lynch et al., 1991). Interestingly, cytopathological changes occur in neurons adjacent to infected microglia rather than in areas with a primary neuronal infection. This observation points to the potentially cytotoxic capacity of infected microglia and show some similarities to observations in HIV encephalitis.

In HIV encephalitis, microglia/macrophages are the primary viral targets (for review see Budka, 1991). *In vitro,* microglia/macrophages rather than any other central nervous system cell type can be easily infected by the virus. *In situ,* a histopathological hallmark of HIV encephalitis is the presence of multinucleated giant cells, which contain large numbers of viral RNA copies per cell (Petito et al., 1986). These cells are thought to be microglia/macrophage-derived and most probably result from the local fusing capacity of HIV. Several pathways could exist for HIV infection within the central nervous system. Virus-infected monocytes/macrophages could enter the brain as "trojan horses" and subsequently spread the infection to resident microglia. The precise entry route of these infected cells is still unclear. However, perivascular cells could be particularly involved, since they are constantly repopulated at a high percentage (30%) by bone marrow cells in the adult central nervous system (Hickey et al., 1992). Bone marrow-derived cells, once infected, could thus enter the brain possibly via a natural homing mechanism.

Alternatively, microglia could become directly infected during the viremia phase. In view of their pronounced cytotoxic potential and the cytopathological changes seen in HIV encephalitis, virus-infected microglia could mediate neuronal injury in AIDS (for review see Lipton, 1992 and Chapter 44, this issue).

RESIDENT MACROPHAGES OF THE PERIPHERAL NERVOUS SYSTEM AND THEIR RESPONSE TO INJURY

Resident macrophages form a prominent, but little recognized component of the peripheral nervous system. They comprise between 1 and 4% of the total endoneurial cell population in the normal peripheral nerve (Oldfors, 1980). These resident peripheral nervous system macrophages have a ramified morphology with processes oriented parallel to the long axis of nerve fibers similar to that of resident microglia in the central nervous system white matter. They show immunophenotypically similarities to central nervous system microglia but also certain differences (Monaco et al., 1992).

Following peripheral nerve injury monocytes/macrophages participate in the phagocytosis of the myelin sheath for example during Wallerian degeneration (Monaco et al., 1992). The involvement of resident peripheral nervous system macrophages in Wallerian degeneration is still a matter of debate. Whereas studies in the C57BL/Ola mouse mutant by Perry et al. (1985) purport to show a major role of hematogenous macrophages (Lunn et al., 1989), there is evidence that resident macrophages could also be involved. *In vitro,* marked myelin phagocytosis occurs, for example, also in nerves without added monocytes (Hann-Bonnekoh et al., 1989), suggesting that resident peripheral nervous system macrophages can be a relevant source of mononuclear phagocytes under certain conditions. The presence of activated macrophages in the injured peripheral nerve is functionally important. Macrophages induce, for example, the synthesis of endoneurial nerve growth factor by secreting interleukin-1 (Lindholm et al., 1987) and could thus be instrumental for regulating peripheral nervous system regeneration.

In addition, activated macrophages, whether derived from the blood or resident in the peripheral nerve, play an important role during nerve inflammation such as in EAN. Macrophages, but not Schwann cells, express MHC class II antigens *in vivo* during EAN (Schmidt et al., 1991). Based on this in vivo demonstration, resident, MHC class II-positive macrophages could qualify as the main intrinsic antigen-presenting cells during EAN. In addition, activated macrophages could cause tissue damage in such immune-mediated peripheral nervous system disorders by elaborating an array of potentially harmful molecules (for review see: Hartung et al., 1992). In summary, the peripheral nervous system is provided with a population of resident macrophages that show certain morphological, functional and immunophenotypical similarities with central nervous system microglia.

FUNCTIONAL REPERTOIRE OF ACTIVATED MICROGLIA

In vivo, a characteristic property of microglia is their swift activation following injury to the brain and their capacity for site-directed phagocytosis. The potentially cytotoxic action of microglia could thus contribute to tissue damage and in turn interference with microglial cytotoxicity might ameleriote the extent of tissue insult. There are two principal ways by which activated microglia can act as cytotoxic cells. First, they can act as phagocytes which involves direct cell-to-cell contact. Second, they are capable of releasing several potentially cytotoxic substances. A summary of the main secretory products of microglia is given in Table 59-1. Cultured microglia release high amounts of hydrogen peroxide (Colton and Gilbert, 1987) leading to neuronal cell death in neuron-microglial cocultures (Thery et al., 1990). As evaluated by flow cytometry they are characterized by a high spontaneous respiratory burst activity, which is even higher than that of control peripheral macrophages, such as thioglycolate-elicited peritoneal macrophages (Banati et al., 1991). Other cytotoxic products of microglia are reactive nitrogen intermediates. Microglia produce high amounts of NO, particularly when stimulated with interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). In addition, microglia release proteinases such as cathepsin B/L and the urokinase-type plasminogen activator (for review see Banati et al., 1993b). This proteolytic activity might also be

TABLE 59-1. *Secretory Products of Microglia*

Reactive oxygen intermediates
Reactive nitrogen intermediates
Excitatory amino acids
Proteinases
Thrombospondin
Amyloid precursor protein (APP)
Quinolinic acid
Cytokines: TGFβ1, TNFα, IL-1, IL-6

involved in tissue remodeling during repair as well as in the cleavage of certain precursor peptides.

Cytokines, such as interleukin-1 (IL-1) and TNF-α, also appear to play a role in microglial cytotoxicity. IL-1 inhibits rat oligodendrocytes *in vitro* (Merrill, 1992). TNF exerts a cytotoxic effect on myelin-forming cells *in vitro*. Interestingly, the lytic action of microglia upon oligodendrocytes can be inhibited by administration of TGF-β1 (Merrill and Zimmermann, 1992), a cytokine, which has in turn been localized in activated microglia themselves *in vivo*. Although there is broad evidence from *in vitro* experiments for a role of cytokines in microglial cytotoxicity, the cytokines relevant for the *in vivo* situation still remain to be identified.

Finally, microglia produce large amounts of glutamate and to some extent also aspartate *in vitro* (Piani et al., 1991). The release of excitatory amino acids points to a crucial role for microglia in NMDA receptor-mediated neuronal injury, which could be particularly important in ischemia.

Several lines of evidence support a key role of microglia during immunopathologies: (1) they are potentially phagocytic cells (Streit and Kreutzberg, 1988; Giulian et al., 1989; Gehrmann et al., 1991a), (2) related to this they have a pronounced cytotoxic potential (Colton and Gilbert, 1987; Thery et al., 1990; Banati et al., 1993b), (3) upon activation they rapidly upregulate the expression of several immunomolecules, such as MHC molecules, the CD4 antigen, and adhesion molecules (for review see Streit et al., 1988), (4) they can effectively present antigen in the molecular context of MHC class II expression to CD4-positive T lymphocytes (Frei et al., 1987; Matsumoto et al., 1992), (5) in the context of MHC class I expression they may become targets of cytotoxic CD8-positive T lymphocytes, and (6) they secrete as well as respond to several cytokines (Frei et al., 1987; Giulian and Ingeman, 1988; Merrill, 1992). *In vitro,* microglia are capable of presenting foreign antigen to CD4-positive T cells in the range of the antigen presenting capacity of professional antigen-presenting cells, such as thymocytes (Frei et al., 1987; Matsumoto et al., 1992). This antigen-presenting function is already maximal under basal, nonstimulated culture conditions and thus surpasses that of astrocytes, which usually have to be primed by IFN-γ to act as antigen-presenting cells (Matsumoto et al., 1992). In addition, rat microglia and, more conspicuously, human microglia show a considerable constitutive expression of MHC class II antigens *in vivo*. Interestingly, in the rat this occurs mainly at central nervous system locations such as lumbar spinal cord, which are primary targets for inflammation. In response to intrathecal application of proinflammatory cytokines, IFN-γ and TNF-α, microglia rather than any other cell type in the central nervous system show a dramatically increased MHC class II expression (Vass and Lassmann, 1990). All these properties make the microglia an important immuneffector cell in the brain.

POSSIBLE SIGNALS FOR MICROGLIAL PROLIFERATION AND ACTIVATION *IN VIVO*

While microglial proliferation and activation are common phenomena in the injured nervous system, little is known about how these processes are regulated *in vivo*. Cytokines appear to be involved in these mechanisms. Colony-stimulating factors (CSFs) are strong mitogens for microglia *in vitro* and furthermore influence their morphological and functional differentiation (Giulian and Ingleman, 1988; Hao et al., 1990, Suzumura et al., 1990). This group of factors consists of three main members: multi-CSF (also known as IL-3), granulocyte macrophage CSF (GM-CSF) and macrophage CSF (M-CSF) (also known in the mouse as CSF-1). While the role of multi-CSF or IL-3 is still debated *in vitro* and *in vivo,* GM-CSF and M-CSF clearly enhance microglial proliferation *in vitro*. These factors act through specific receptors on the microglial surface. The MCSF receptor is a single-chain tyrosine kinase related to the platelet-derived growth factor and the stem cell factor receptor (c-kit), whereas the GM-CSF receptor consists of a heterodimer belonging to the family of hematopoetic growth factor receptors. Following injury activated microglia upregulate within 24 hours the expression of both the M-CSF and the GM-CSF receptor (Raivich et al., 1991a). The *in vivo* sources of the respective ligands, M-CSF and GM-CSF, still remain open. Based on *in vitro* observations, activated microglia as well as reactive astrocytes may be potential sources for these microglial mitogens. An *in vivo* role of CSFs for microglial proliferation is supported by recent experiments by Fedoroff and colleagues and Raivich and colleagues in the opi mouse. Opi mice have a frameshift mutation in the M-CSF gene and, most likely due to the absence of M-CSF, facial nerve axotomy does not lead to microglial proliferation (Raivich et al., 1994).

Studies in experimental neuropathology show that the microglia rather than any other cell type in the brain responds to injury. Microglial activation may even precede pathological changes or occur in the absence of obvious neuropathic changes. Thus microglia appear to respond rapidly to even subtle changes in their microenvironment. Microglia dis-

play a unique potassium channel pattern *in vitro* (Kettenmann et al., 1990), which may make them more sensitive to changes in extracellular potassium than any other cell type in the brain. In view of this channel pattern of microglia, it has been postulated that microglia could be extremely susceptible to neuronal depolarization and associated changes in extracellular potassium. In the model of cortical spreading depression (CSD) recurrent cortical depolarizations can be elicited by the transient, extradural application of high potassium. A microglial reaction with an immunocytochemical peak 24 hours after the end of CSD occurs in the entire cortex ipsilateral to the side of CSD induction but not in deeper brain areas (Gehrmann et al., 1993b). This microglial reaction occurs in the absence of neuropathological damage except for the direct site of potassium application. Although CSD induces several metabolic changes other than increased potassium fluxes across membranes, for example, release of glutamate, hypoperfusion, increased glucose consumption, the microglial reaction after CSD provides further evidence that potassium might be involved in regulating microglial activation *in vivo*.

In addition, neurotransmitters may act as signaling substances on microglia. Most information on this topic comes from *in vitro* studies, mostly electrophysiological measurements. While classical neurotransmitters such as GABA or glutamate, which act on astrocytes or oligodendrocytes have no apparent effect on microglia, acetylcholine and purines might be involved. Carbachol, a muscarine acetylcholine agonist, leads to increased calcium fluxes in cultured microglia. Adenosine triphospate (ATP) a P2-purinoreceptor agonist changes the electrophysiological properties of microglia by promoting depolarization (Kettenmann et al., 1993). Since ATP can be released as a cotransmitter (e.g., with norepinephrine or acetylcholine) and is furthermore released by several other cell types during tissue injury, ATP could be involved in microglial activation.

CONCLUSIONS

In summary, microglia form a network of resident central nervous system immuneffector cells. Microglial activation occurs after injury or changes in the microenvironment, often either before or without detectible changes in other cell types. The mechanisms by which microglial activation and proliferation are regulated *in vivo*, however, still is obscure. More insight into how these processes are regulated *in vivo* might provide a better understanding of how immune reactions in the central nervous system could be manipulated.

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60 Glial cells and axonal regeneration in the central nervous system

CLAUDIA A. O. STUERMER

Unlike neurons of amniotic vertebrates, such as mammals, birds, and reptiles, those in the central nervous system of fish and amphibians are capable of spontaneously regenerating their axons (for review see Gaze, 1970). In fish, this applies to several central nervous system fiber tracts and includes among others the visual pathway and descending tracts of the spinal cord (Bunt and Fill-Moebs, 1984; Wanner and Stuermer, 1992; Sharma et al., 1993). Yet with regard to axon-glial cell interactions, the retinal projection to the midbrain tectum—and hence regeneration of retinal ganglion cell axons through the optic nerve and tract up into the tectum—has been more closely analyzed than other central nervous system tracts of the fish. The axonal response to injury in the amphibian visual system has also been studied extensively; a substantial proportion of retinal ganglion cells (between 30 and 70%) in this system regenerate their axons (for review see Stelzner et al., 1986). However, since the contribution of amphibian central nervous system glia to the success or failure of axonal regeneration remains elusive, this chapter is predominantly concerned with the interplay of regenerating retinal ganglion cell axons and glial cells in the fish visual pathway.

Preceding chapters have discussed the reasons for the failure of axonal regeneration in the mammalian central nervous system, whose oligodendrocytes and myelin carry axon growth-inhibiting molecules on their surface (Caroni and Schwab, 1988a; Schwab and Caroni, 1988) and where astrocytes forming a so-called glial scar acquire nonpermissive properties (for review see Bovolenta et al., 1991). We have learned, however, that in the rat, a few central nervous system axons do regenerate successfully under certain circumstances. *In vivo*, some previously injured axons regrow when the oligodendrocyte-associated axon growth inhibitor is neutralized by specific antibodies (Schnell and Schwab, 1990), when oligodendrocytes are absent or sparse (Savio and Schwab, 1990; Berry et al., 1992) or when the optic nerve is surgically replaced by a peripheral nerve graft with living Schwann cells (Benfey and Aguayo, 1981; review: Bray et al., 1992). *In vitro*, segments from the adult rat retina will extend axons when removed from their normal optic nerve glia environment, as for instance, when the retinal segments are explanted onto a substrate consisting of Schwann cells or immature astrocytes (Bähr and Bunge, 1989, 1990). The number of rat retinal ganglion cells that do regenerate their axons *in vivo* or *in vitro* is, however, small and does not exceed more than 5% of the total number of retinal ganglion cells (Villégas-Pérez et al., 1988).

In contrast to the rat, retinal ganglion cell axonal regeneration in fish occurs spontaneously and does not require surgical aids, such as grafts, or the neutralization of possible inhibitors. Nor are there astrocytic glial scars in fish. Furthermore, in fish all or nearly all retinal ganglion cells, not just a fraction, are capable of regenerating their axons (Meyer et al., 1985). Finally, axonal regeneration in fish leads to the restoration of a retinotopic map in the visual target center, the optic tectum, and to recovery of normal vision and visuomotor behavior (for review see Gaze, 1970).

While recovery of function after optic nerve injury in fish has been known for several decades, studies on the particular role of the glial cells for the success of axonal regeneration were only recently undertaken. Thus far, the characteristics of glial cells that are partners to neurons in the central and peripheral nervous systems are far better analyzed in mammals than in fish. With the knowledge that Schwann cells of the mammalian peripheral nervous system allow and support axonal regeneration and that oligodendrocytes and central nervous system myelin inhibit axonal growth, two questions arose: Does the fish central nervous system contain typical oligodendrocytes? How does the myelin-forming glial cells of the fish central nervous system differ from those in the peripheral nervous system? (Jeserich and Rauen, 1990).

Before turning to the issue of glial cell contribution to axonal regeneration in the fish optic nerve,

the characteristics of myelin and myelin-forming cells of fish central and peripheral nervous systems are briefly summarized.

CENTRAL NERVOUS SYSTEM AND PERIPHERAL NERVOUS SYSTEM MYELIN AND MYELIN-FORMING CELLS IN TELEOSTS

Studies of the phylogeny of myelin proteins have detected similarities, but also some striking differences, in the myelin protein composition of fish and mammals (Waehneldt and Jeserich, 1984; Jeserich and Waehneldt, 1986a, 1986b, 1992). Fish peripheral nervous system and central nervous system myelin contains, as does myelin in mammals, substantial amounts of myelin basic protein (MBP). However, the unglycosylated hydrophobic proteolipid protein (PLP) of the mammalian central nervous system myelin appears to be absent from fish central nervous system myelin, or at least not expressed to such an extent as in mammals. Instead of PLP the fish central nervous system myelin exhibits two glycosylated, *hydrophobic proteins*, IP1 and IP2 with molecular weights of 23 kD and 27 kD, respectively, which are recognized by the antibody 6D2. These two proteins are not found in mammalian central nervous system myelin. Interestingly, the IP1 and IP2 proteins are recognized by antibodies against P0, the structural myelin protein of the mammalian peripheral nerve system. P0 is defined as a cell adhesion molecule of the immunglobulin superfamily (Sakamoto et al., 1987; Williams and Barcley, 1988) and appears to function in the peripheral nervous system as PLP does in the central nervous system. The N-terminal amino acid sequence of IP1 and IP2 has similarities to that of mammalian P0 (Schliess and Stoffel, 1991; Jeserich and Waehneldt, 1992), but further molecular characterizations of IP1 and IP2 have not been carried out.

6D2 immunoreactivity is also associated with fish peripheral nervous system nerves. Still, this does not imply that the fish central nervous system myelin-forming cells are identical to myelin-forming cells of the peripheral nervous system or that they perhaps even represent Schwann cells. Earlier electron microscopic examinations of axonal regeneration in the goldfish oculomotor nerve demonstrated clearly that peripheral nervous system axons are associated with and remyelinated by cells with typical characteristics of Schwann cells (Scherer and Easter, 1984). Myelin-forming cells in the fish peripheral nervous system show a one to one relationship with myelinated axonal segments, as is typical for Schwann cells of the mammalian peripheral nervous system (Scherer and Easter, 1984). Furthermore, their mammalian peripheral nervous system counterparts, like these fish Schwann cells, produce a basal lamina that is missing from oligodendrocytes of both fish and mammals. Moreover, recent data have demonstrated immunocytochemically the expression of nerve growth factor (NGF) receptors by fish peripheral nervous system-derived Schwann cells in culture (Bastmeyer and Stuermer, 1992). Much as in mammals, anti-NGF receptor immunoreactivity is not found on the central nervous system-derived glia cells of fish.

There is another protein in fish, the 36k protein, that is specific for fish central nervous system myelin and absent from the peripheral nervous system (Jeserich and Rauen, 1990), substantiating a clear distinction between central nervous system and peripheral nervous system myelin and myelin-forming cells in fish. The central nervous system/peripheral nervous system distinction was further supported by electron microscopic examinations. These electron microscopic studies have revealed a close structural similarity between myelin-forming cells in the fish central nervous system with mammalian oligodendrocytes, in that the cells extended myelinating processes to nerve fibers in their vicinity (Jeserich and Waehnelt, 1986b). This classifies them as oligodendrocytes.

It is clear for mammals and birds that Schwann cells and oligodendrocytes derive from different lineages. The former are neural crest derivatives and oligodendrocytes arise from central nervous system subventricular zones. Glial precursor cells giving rise to oligodendrocytes have been characterized by a ganglioside epitope defined by the antibody A2B5 (Raff et al., 1983).

Jeserich and Stratmann (1992) isolated central nervous system glial cells from the developing trout brain. In addition to glial fibrillary acidic protein (GFAP)-positive astrocytes, cells with multiple processes morphologically resembling oligodendrocytes also developed in these cultures (for review see Jeserich et al., 1990). The latter in fact differentiated from bipolar precursor cells, which exhibit the A2B5 epitope (Jeserich and Stratmann, 1992). Oligodendrocytes isolated from trout at various developmental stages express myelin proteins in a chronological sequence. At stage 28, IP2 and galactocerebroside (GalC) are present; at stage 30, 36k is expressed, and at stage 32, IP1. MBP was not detected in glial cells in culture but was found in parallel with IP2 in sections from the developing trout brain (Jeserich and Stratmann, 1992). With time in culture, however, trout oligodendrocytes cease to express GalC, 36k, and IP1, but continue to show IP2. When cultured as precursors, however, the A2B5-positive cells do

not differentiate beyond the level of IP2 expression, which signifies the initial step of oligodendroglial development *in vivo* (Jeserich and Stratmann, 1992).

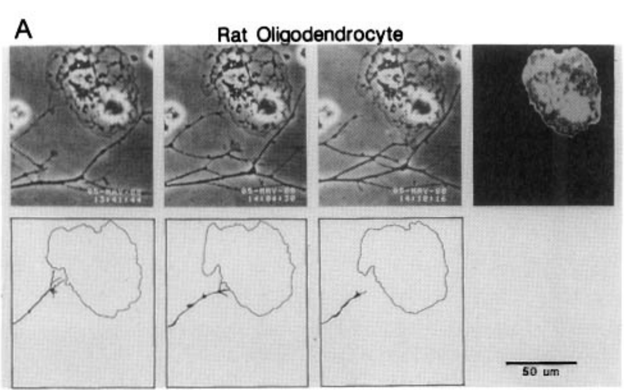
In summary, myelin in fish is formed by typical oligodendrocytes in the central nervous system and by Schwann cells in the peripheral nervous system. However, some of the major central nervous system myelin proteins of fish oligodendrocytes differ from those in mammals. Furthermore, fish oligodendrocytes *in vitro* do not retain or—when plated as precursors—do not spontaneously reach the differentiation stages that they exhibit *in vivo.*

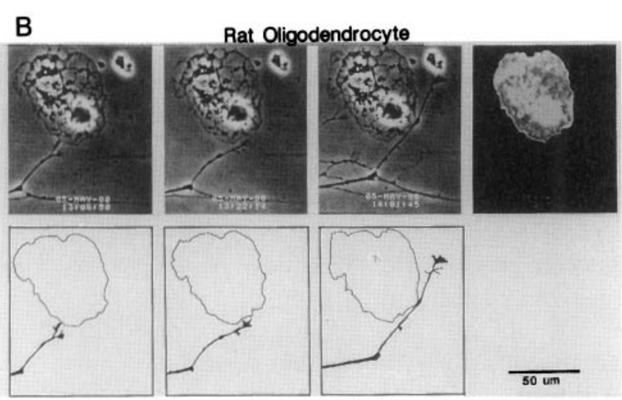
But do oligodendrocytes in the fish central nervous system, particularly in the optic nerve, possess similar growth-inhibiting molecules as mammalian oligodendrocytes? One could argue that successful axonal regeneration would still be possible in the presence of inhibitors if fish retinal axons were able to overcome the inhibitory properties of these glial cells by producing, for instance, substances such as proteases that would inactivate molecules or if fish axons lacked the sensitivity (receptors) for inhibitors. Alternatively, successful regeneration is perhaps to be expected if fish oligodendrocytes and central nervous system myelin do not express such inhibitory molecules.

RESPONSE OF FISH AXONS TO RAT AND FISH CENTRAL NERVOUS SYSTEM MYELIN AND OLIGODENDROCYTES

Whether fish retinal ganglion cell axons are sensitive to the mammalian central nervous system myelin/oligodendrocyte inhibitor was addressed in the first of a series of cross-species coculture assays.

The first assays examined the interaction of regenerating fish retinal axons with mammalian oligodendrocytes. Rat optic nerve oligodendrocytes were cocultured with fish retinal explants (Bastmeyer et al., 1991), and the contact of the growing fish axons with the mammalian glial cells was monitored with time-lapse videomicroscopy. When the fish axons touched the mammalian oligodendrocytes, the growth cones collapsed (Figure 60-1A) and pulled back, or the axons grew around the cells but did not cross them (Figure 60-1B). In the presence of the specific antibody IN1 which neutralizes the mammalian inhibitor (Caroni and Schwab, 1988b), however, the fish axons grew over the mammalian oligodendrocytes (Figure 60-2). These results indicated that regenerating fish retinal axons are indeed sensitive to the mammalian oligodendrocyte/myelin-associated inhibitory proteins. In fact, the fish axons reacted in a manner that was identical to the behavior





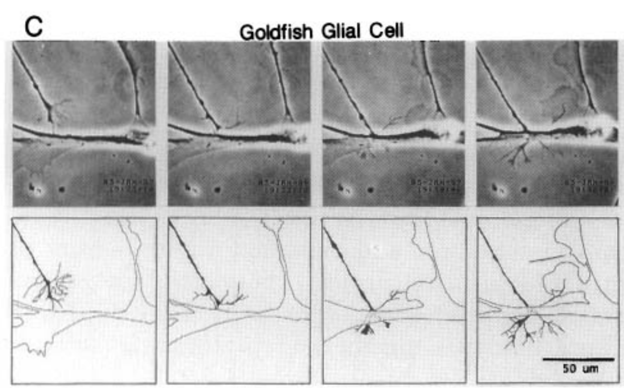


FIG. 60-1. Selected frames from time-lapse video sequences and camera lucida tracings showing (A,B) the encounter of goldfish retinal growth cones with mammalian oligodendrocytes, and (C) a goldfish optic nerve oligodendrocyte *in vitro*. The growth cones either collapse upon contact with a mammalian oligodendrocyte (Figure A) or grow around the cell (Figure B), but they cross fish oligodendrocytes freely (Figure C). The mammalian oligodendrocyte was labeled with the antibody GalC (fluorescence image at *right*).

FIG. 60-2. Contacts of goldfish retinal axons with a highly branched rat oligodendrocyte in the presence of MAB IN-1 observed with time-lapse videomicroscopy. (**A,B,C**) One axon grows over the oligodendrocyte (*arrows*) and crosses several of its processes. Another axon (*arrowheads*) grows along the cell perimeter. (**D**) The oligodendrocyte was identified with GalC (fluorescence image). Time in hours, minutes, and seconds is shown at the lower right of each frame. Scale bar, 20 μm for Figures A–D.

of mammalian axons upon encountering mammalian oligodendrocytes and central nervous system myelin (Fawcett et al., 1989; Bandtlow et al., 1990). The sensitivity of fish retinal ganglion cells to the mammalian inhibitor was confirmed by offering mammalian central nervous system myelin as a substrate for the growth of fish retinal axons. On mammalian central nervous system myelin, fish retinal ganglion cells failed to extend axons (Bastmeyer et al., 1991).

The next step was an evaluation of the reaction of growing axons to oligodendrocytes derived from the fish optic nerve and to fish central nervous system myelin. This required isolating these glial cells and identifying them in culture.

Glial cells were obtained from the regenerating fish optic nerve and tract either by dissociation or by explanting segments of the nerve from which glial cells emigrated. One type of cell morphologically resembled astrocytes and exhibited a high density of anti-GFAP-positive fibrils. These cells never expressed any myelin marker proteins. They divided in culture, but stuck together in typical clusters (Hoppe et al., 1991).

The other glial cell type was typically bipolar in appearance with membraneous veils over its entire surface and could be identified as belonging to the oligodendrocyte lineage. When optic nerves were dissociated and the isolated cells probed with antibodies, some of these cells showed anti-36k and -6D2 immunoreactivity within a few hours after plating. At later times in culture, these glial cells, as well as elongated ones emigrating from nerve explants, divided and, within a few weeks, formed a networklike carpet.

Unlike mammalian oligodendrocytes that differentiate in culture and acquire further markers, such as GalC immunoreactivity, fish optic nerve/tract-derived oligodendrocytes remain GalC-negative. Fish optic nerve/tract oligodendrocytes in early dividing cultures expressed GFAP fibrils, which extended into the elongated processes. However, staining with anti-GFAP was always weaker than with fish astrocytes, and was transient and coexistent with myelin markers IP2 and 04 immunoreactivity. With time in culture, oligodendrocytes lost GFAP and gained in addition to 04 increased 6D2 immunoreactivity (Bastmeyer et al., 1991). They did not show spontaneous 36k immunoreactivity, but acquired this myelin protein when they were cocultured with axons (Bastmeyer et al., 1994). Furthermore, in these cocultures with retinal axons, the optic nerve/tract oligodendrocytes began to enwrap axons, one cell associated with several axons, in a manner characteristic of the onset of myelination (Figure 60-3) (Bastmeyer et al., 1993). Thus, despite their otherwise unusual characteristics, the goldfish optic nerve/tract-derived glial cells are to be considered counterparts of mammalian oligodendrocytes.

When fish retinal growth cones encountered fish oligodendrocytes in a coculture, the axons readily crossed them (Figure 60-1C). This implies the absence of growth inhibitors. But would fish oligodendrocytes produce inhibitors upon further differentiation, as they do *in vivo* but not *in vitro*? If so, the

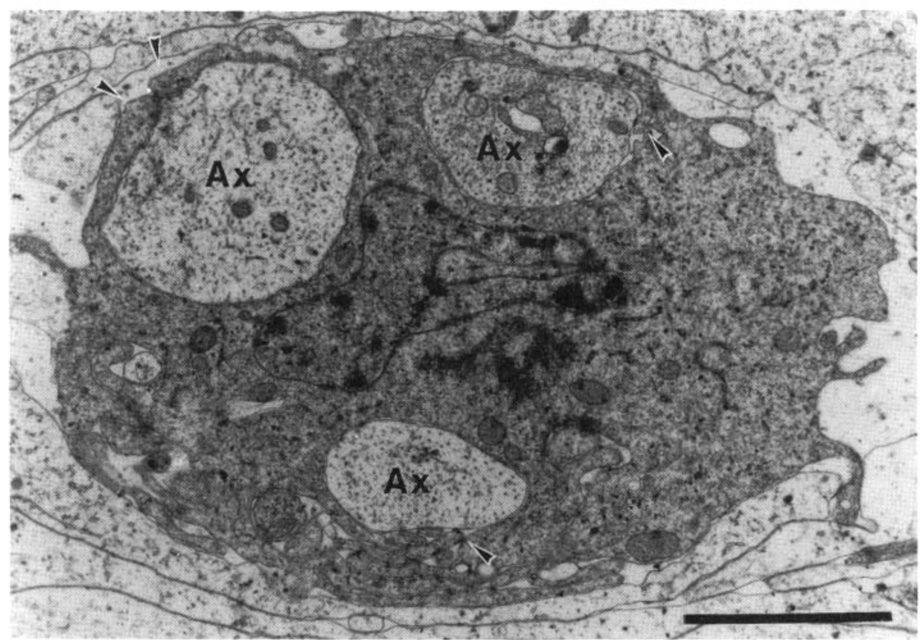


FIG. 60-3. Fish oligodendrocyte ensheathing rat retinal axons. This electron micrograph was taken from a 4-week-old coculture of fish glial cells and rat retinal axons. The dark cytoplasm and the idented nucleus identify the cell as an oligodendrocyte. The cell has totally surrounded the axons (*Ax*) and has formed vermicular processes (three of which are marked by *arrowheads*) around them. The axons are identified by their electronlucent cytoplasm, neurofilaments, microtubules and the absence of ribosomes. Scale bar: 2 μm.

inhibitory influence on axon growth should be found in the optic nerve myelin or central nervous system myelin of adult animals.

When fish retinal explants were grown on a substrate of fish central nervous system myelin, axons extended and were capable of elongating on the myelin substrate. A similar observation was made when rat axons were confronted with fish central nervous system myelin (Vanselow et al., 1990). Even for rat retinal axons, fish central nervous system myelin was a growth-permissive substrate. In the reverse experiment, with rat central nervous system myelin as substrate, outgrowth of axons from fish retinal explants and rat axons failed (Bastmeyer et al., 1991). These results are in accordance with earlier data, which already had indicated the absence of axon growth inhibitors from fish optic nerve myelin. Carbonetto and colleagues (1987) showed that rat dorsal root ganglia (DRG) would extend axons when offered thin cryostat sections of fish optic nerves as a substrate. These neurons, however, would not grow on sections of mammalian central nervous system nerves.

Taken together, the outcome of these experiments is consistent with the notion that fish oligodendrocytes and central nervous system myelin lack the axon growth-inhibiting proteins of mammals, and fish retinal axons, much like axons of higher vertebrates, are sensitive to inhibitors of mammalian central nervous system myelin and oligodendrocytes. In other words, one reason for the success of axonal regeneration in the fish optic nerve could be the absence of inhibitors and thus the growth-permissive properties of the fish oligodendrocytes and the fish central nervous system myelin.

To test whether this conclusion from *in vitro* studies would also apply to the *in vivo* situation, we attempted to identify the elements associated with the regenerating growth cones in the fish optic nerve (Strobel and Stuermer, 1994). The growth cones of the regenerating axons were labeled with horseradish peroxidase from the eye. From ultrathin serial sections, the growth cones and the elements with which they were in intimate contact were reconstructed. Regenerating growth cones were found in direct membrane to membrane association with myelin debris (Strobel and Stuermer, 1994), a situation that would not be encountered in the mammalian central nervous system. They also were in close contact with a type of an oligodendrocyte precursor or a microglial cell based on criteria derived from work on mammalian glial cells and data from fish studies (Dowding et al., 1991). Growth cones were also seen in contact with other cell types of the regenerating optic nerve, indicating that they may not have a strong preference for a specific type of cell. However, these observations support the idea that myelin/oligodendrocyte-associated inhibitory molecules which repel or negatively affect regrowing fish retinal axons may be absent from the goldfish optic nerve *in vivo*.

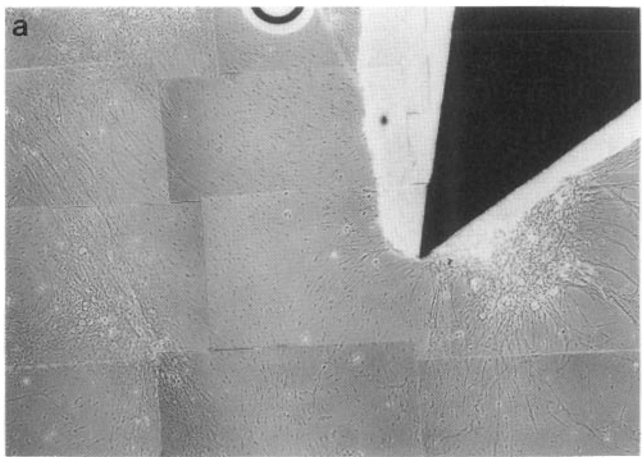
GROWTH OF FISH AND RAT AXONS ON FISH OLIGODENDROCYTES *IN VITRO*

Further experiments extended this conclusion: beyond their lack of inhibition influence, fish optic-

nerve-derived oligodendrocytes are—at least in their differentiation state observed *in vitro*—extremely growth supportive. Evidence fostering this view include, first, that the number of axons emerging from fish retinal explants on a substrate of fish oligodendrocytes was higher (Bastmeyer et al., 1993) than on any other substrate used so far (Vielmetter et al., 1990). Moreover, when retinal explants were placed on untreated plastic coverslips they failed to extend axons. In the presence of fish oligodendrocytes, however, a substantial number of axons emerged from the retinal explant, suggesting that these glial cells indeed promote axonal growth (Bastmeyer et al., 1993; for review see Stuermer et al., 1992). Furthermore, the retinal growth cones appeared to grow preferentially in close contact with the oligodendrocytes, rendering the impression that these glial cells carry surface-associated molecules recognized by the growth cones as favorable substrates for their elongation. Some axons also grew in areas where the oligodendrocytes had been but from which they had migrated away (Stuermer et al., 1992), giving the impression that the glial cells may leave some growth-promoting molecules on the coverslip.

These observations stimulated another cross-species experiment to address the following question. Since the inhibitory proteins of the mammalian oligodendrocytes negatively affected the fish retinal axons, would the growth-supportive properties on the surface of the fish oligodendrocytes exert a positive influence on injured rat retinal axons? Segments from the adult rat retina were explanted onto the network-like carpet of fish oligodendrocytes (Bastmeyer et al., 1993; for review see Stuermer et al., 1992). These cocultures necessitated conditions that were a compromise between the requirements of warm- and cold-blooded vertebrates. Thus, cultures were grown at a temperature of 28°C but without the additional O_2/CO_2 supply commonly used for rat neural tissue. Adult rat retina explants usually fail to regenerate axons under such conditions. Yet in the presence of the fish oligodendrocytes, a large number of axons emerged from the rat retinae (Figure 60-4) and reached lengths of up to 4 to 5 mm. These rat axons grew in close contact with the surfaces of the fish oligodendrocytes and avoided fish oligodendrocyte-free regions. Thus, fish oligodendrocytes *in vitro* apparently possess cell surface-associated molecules that stimulate axonal regeneration. These molecules not only promote axonal regeneration of fish retinae but also of adult rat retinae.

A candidate molecule that may contribute to the growth supportiveness of fish oligodendrocytes *in vitro* was identified by the goldfish-specific monoclonal antibody (MAb) E 587. E 587 is directed against a 200 kD glycoprotein (Vielmetter et al., 1991), related to the class of the L1 family of cell adhesion molecules (Rathjen et al., 1987; Lagenaur and Lemmon, 1989). While usually distributed evenly on the surface of the fish oligodendrocytes, this protein appears to cluster when processes of two cells overlap (Figure 60-5A–C) (Bastmeyer et al., 1993). The E 587 antigen is also found on regenerating fish retinal axons (Vielmetter et al., 1991), an



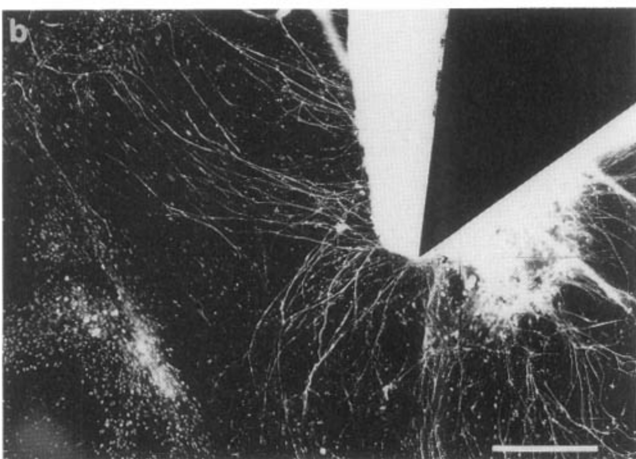
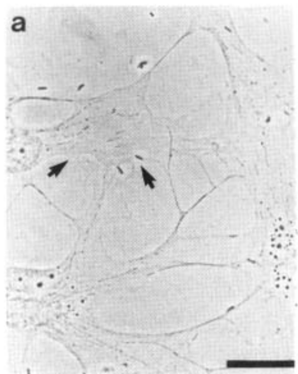
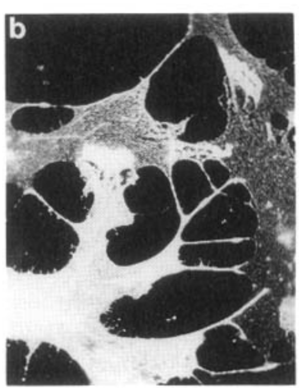
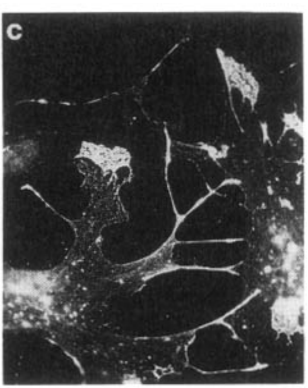


FIG. 60-4. A segment of the retina of an adult rat, explanted onto fish glial cells. Photomicrograph of the coculture, (**A**) in phase contrast, and (**B**) after staining the axons with the antineurofilament antibody SMI 31. (**A**) Rat retinal explant attached to a filter (*black triangle*). *Arrowheads* mark boundaries between purely oligodendrocyte territory and clusters of astrocytes, into which a few oligodendrocytes have penetrated. The *arrows* point to a cell free region. The cells at the lower right are glial cells which have emigrated from the rat retinal explant. (**B**) The rat retinal axons extend along the fish oligodendrocytes and can be seen to follow the elongated orientation of the oligodendrocytes. The rat axons rarely cross regions free of glial cells and only a few grow onto the fish astrocyte cluster. (Unspecific staining of the nuclei of the cells by SMI 31.) Scale bar for both figures, 500 μm.







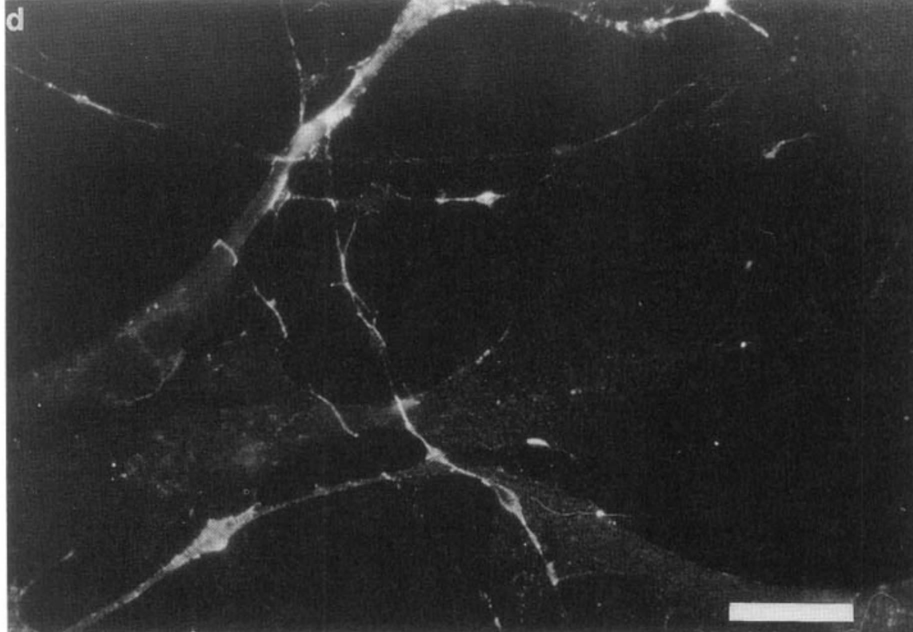


FIG. 60-5. Fish oligodendrocytes in culture. (**A**) Phase contrast. *Arrows* point to overlapping processes of oligodendrocytes. (**B**) Same area showing immunoreactivity to 04, which labels the entire glial cell surface. (C) Same area showing E 587 immunoreactivity, which is predominantly accumulated along areas of contact between two cells. (D) When fish retinal axons grow on the surface of fish oligodendrocytes, the E 587 antigen accumulates at contact sites between the axon and the cell. Where growing on the laminin-coated glass coverslip, the axons exhibit very little MAb E 587 immunoreactivity (*arrows*) but are brightly stained where they grow on the oligodendrocytes (*arrowhead*). Scale bar in Figure A applies to all figures: 20 μm.

interesting aspect that will be considered more closely below. When the regenerating axons grow along the surface of the oligodendrocytes, the area of contact between the axon and the cell again exhibits a striking contact-dependent accumulation of the antigen (Figure 60-5D) (Stuermer et al., 1992). This contact-dependent clustering implies involvement of this protein interaction of the axons and the glial cells. This has recently been confirmed in ongoing experiments using the E 587 antibody to interfere with function (Giordano et al., 1993); Bastmeyer, unpublished results). Whether E 587 antigen contributes to retinal axonal regeneration along oligodendrocytes *in vivo* awaits the outcome of further experiments.

RESPONSE OF THE FISH RETINAL GANGLION CELLS TO OPTIC NERVE SECTION: REEXPRESSION OF GROWTH-ASSOCIATED CELL SURFACE PROTEINS

To regenerate successfully, a neuron whose axon has been cut must reinduce the relevant intracellular mechanisms and synthesize the necessary proteins to support axonal growth. Moreover, the long-range

pathfinding functions that operate during development must be reactivated, as well as the conditions required for the reestablishment of the appropriate connections in the target. At the molecular level, these functions are only beginning to be elucidated. The success of retinal axon regeneration and the restoration of vision in fish implies that the fish ganglion cells and their targets possess the required features.

Earlier studies have demonstrated that fish retinal ganglion cells enlarge and increase their RNA and protein synthesis manifold after optic nerve transection (McQuarrie and Grafstein, 1981). Among the proteins whose quantities are elevated in response to injury, a few were identified, such as the growth-associated protein Gap 43 (for review see Skene, 1991), cytoskeletal proteins, and proteins needed for the addition of new membranes (Perry et al., 1985, 1987; Benowitz et al., 1991).

The extent of this injury-induced reexpression of growth-relevant proteins in the fish retina exceeds that of the rat retinal ganglion cells manifold. Gap 43, for instance, reappears on a limited number of rat ganglion cells and Gap 43-positive retinal ganglion cells correlate numerically with rat retinal ganglion cells, which, under favorable conditions, would regenerate an axon (Doster et al., 1991). However, Gap 43 reappears only on these rat retinal ganglion cells when the optic nerve is severed close to the eye. If a lengthy piece of optic nerve remains connected to the eye (as is the case if the optic nerve crush is made closer to the brain) the injured retinal ganglion cells fail to exhibit Gap 43 (Doster et al., 1991; Skene, 1991). Skene raised the interesting possibility that the presence of oligodendrocytes which express growth inhibitors may supress synthesis of growth-associated proteins such as Gap 43 via a retrograde signal along the axons (Skene, 1989, 1991). Cuts close to the eye and separation from the optic nerve oligodendrocytes releases this suppression and allows renewed protein synthesis—at least in a few "competent" neurons. This view is consistent with the immediate upregulation of protein synthesis in injured retinal ganglion cells of fish and the absence of growth inhibitors from fish optic nerve oligodendrocytes.

Along this line, several recently identified cell surface-associated proteins were found to be reexpressed during fish retinal ganglion cell axonal regeneration. Three of these molecules belong to the class of cell adhesion molecules (E 587 antigen being one of them) and may play a role in axonal pathfinding and target reinnervation (Wehner and Stuermer, 1989; Bastmeyer et al., 1990; Stuermer, 1991b; Vielmetter et al., 1991; Paschke et al., 1992). They were discovered using monoclonal antibodies (MAbs), and defined as growth-associated cell surface proteins, since the MAbs always and only stained axons that were in a state of growth. These included, for instance, all retinal axons in the goldfish embryos, and those newly added in the adult (Stuermer et al., 1992). More mature axons had lost their MAb immunoreactivity but all regenerating retinal axons in the retina, in the optic nerve and tract, and in the retinorecipient layers of the tectum reexpressed these proteins on their surfaces (Wehner and Stuermer, 1989; Bastmeyer et al., 1990; Vielmetter et al., 1991; Paschke et al., 1992). Thus, retinal ganglion cells in fish retain their ability to reexpress growth-associated cell surface proteins —thought to assist axonal pathfinding—throughout adulthood. Reexpression is induced when the axons are severed and then regenerate. Furthermore, the ganglion cells are capable of downregulating the expression of these proteins once appropriate connections with the target have been reformed.

The regulated reexpression of the growth-associated proteins by fish retinal ganglion cells conforms with Skene's hypothesis. Because inhibitors are absent from fish optic nerve oligodendrocytes and myelin there is no suppression of renewed protein synthesis. Indeed, the presence of growth-stimulating molecules on fish glial cells could function as a positive feedback signal for the fish retinal ganglion cells and thus enhance the reexpression of growth-associated proteins and the reextension of severed axons. Experiments for testing this hypothesis are underway and may determine whether such postulated feedback pathways from glial cells to neurons exist.

ASTROCYTES IN THE REGENERATING FISH RETINOTECTAL PATHWAY

A possible role of astrocytes in axonal regeneration in the fish optic nerve/tract and tectum has been proposed (Murray, 1976; Nona et al., 1989; Levine, 1991), but the axon-astrocyte interplay during axonal regeneration is not understood. One reason is that the common marker for astrocytes, GFAP, is not expressed by all presumed astrocytes in the fish visual pathway, nor is it expressed exclusively by astrocytes. Even oligodendrocytes from regenerating optic nerve/tract in early stages of their differentiation contain anti-GFAP positive fibrils (Bastmeyer et al., 1991) and may or may not transiently produce cytokeratins. Fish glial cells exhibit additional filament proteins (Jones et al., 1986; Maggs and Scholes, 1986), one of which has been identified as a cytokeratin (Giordano et al., 1989). It is not known whether cytokera-

tin(s) are contained solely in astrocytes or in both astrocytes and undifferentiated oligodendrocytes.

Moreover, the filament protein expression pattern in fish optic nerve/tract glial cells changes after optic nerve injury and changes again when the axons regenerate and grow toward the tectum (Maggs and Scholes, 1986; Levine, 1989; Levine, 1991; Stafford et al., 1990). As more of these fish glial filament proteins are being characterized on a molecular level, and new probes are being generated, the cell type (and its differentiation stage) that expresses these proteins will probably be determined in the near future.

In optic nerve/tract glial cultures such as those described above, both GFAP-positive astrocytes and oligodendrocytes proliferated, but the astrocytes formed small clusters interspersed among the network of oligodendrocytes (Bastmeyer et al., 1993). As discussed earlier, regenerating axons—whether from fish RGCs or rat RGCs—grew in intimate contact with the surface of the fish oligodendrocytes. Surprisingly, when the axons met the astrocyte clusters they grew around them rather than crossing them (Bastmeyer et al., 1993). Thus, fish axons, as well as those of the rat, preferred the surface of the fish oligodendrocytes over astrocytes for their growth. Whether this unexpected result is solely characteristic of these cultures or also applies to the *in vivo* situation remains to be clarified.

In this respect, it is interesting to note that upon optic nerve crush the crush zone of roughly 500 μm remains free of glia filament-expressing glial cells for weeks (Stafford et al., 1990; Levine, 1991). Elongated GFAP-positive cells on both edges of this zone are oriented parallel to the direction of growth of the axons. With time, similar cells populate the crush zone. Although the identity of these cells and their role in axonal regeneration remains elusive, their presence at least substantiates the earlier findings that there is no such structure as a glial scar in fish.

Hopkins et al. (1985) demonstrated a generally elevated level of laminin immunoreactivity in sections of the regenerating goldfish optic nerve. The identity of the cells that would produce this growth-promoting extracellular matrix molecule is not known. However, this together with the findings that fish central nervous system myelin and oligodendrocytes lack axon growth inhibitors and that fish oligodendrocytes at least in culture, carry an L1-like molecule on their surfaces, support the notion that severed retinal axons and newly formed growth cones in fish find a hospitable rather than hostile environment for their regenerative growth.

CONCLUSION

In contrast to mammals, central nervous system myelin and oligodendrocytes of the fish visual pathway perhaps do not carry axon growth-inhibiting proteins, but are growth-permissive instead. The absence of growth inhibitors is likely to be one of the reasons that fish retinal ganglion cells can so successfully regenerate their axons. In fact, fish oligodendrocytes of differentiation stages that they reach in culture express axon growth-promoting molecules and allow the regrowth along their surface of not only fish axons but also rat retinal ganglion cell axons. Whether fish oligodendrocytes exert a growth-stimulating effect on injured axons *in vivo*, however, needs to be further investigated.

It appears reasonable to assume that the reexpression of sets of growth-associated proteins by injured neurons are a necessary prerequisite for axonal regrowth. In fish, at least the upregulation of protein expression by all retinal ganglion cells always occurs in parallel with axonal regeneration. It will be interesting to learn whether and how the properties of the glial cells in the vicinity of the axons influence —positively or negatively—protein expression of the injured neurons.

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XII Glia and Disease

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61 Brain edema

HAROLD K. KIMELBERG

CELLULAR AND VASOGENIC EDEMA

It is well-recognized that brain edema is an important component of the pathophysiology of stroke and traumatic brain injury, as well as a number of other pathological states. Brain edema has been broadly classified into two major categories (Klatzo et al., 1984): vasogenic edema and cellular or cytotoxic edema. These are illustrated in the scheme shown in Figure 61-1. It should, however, be emphasized that the term *edema* is best restricted to conditions in which there is always a *gain* of water, with or without cell swelling. Such water has to come from outside the central nervous system crossing the endothelial cells of the blood-brain barrier, as shown in Figure 61-1. The blood-brain barrier allows water to pass, but excludes polar substances, unless there are specific carrier systems present (Davson, 1976; Goldstein and Betz, 1986). The general term for a gain of water by the brain from the blood is *vasogenic edema.* There is commonly an increased extracellular space, but cell swelling can also occur (Klatzo et al., 1984) (see Figures 61-4 and 61-6 and the section *Volume Measurements In Situ* below). However, cell swelling can also occur without a gain of water when there is simply a *shift* of water from the extra- to intracellular space. This can occur under nonpathological conditions, for example with intense neuronal activity (Van Harreveld and Schade 1962; Ransom et al., 1985). As shown later in Figure 61-4, pure cellular swelling may also be a first step in brain edema, which subsequently occurs during the reperfusion phase after ischemia (Hossman, 1971). Water gain will increase volume within the closed cranial cavity, leading to an increased intracranial pressure (ICP), which can be readily measured by pressure transducers. Increased intracranial pressure will also occur with increased intracranial blood volume, which is a serious problem in traumatic brain injury when the intracranial compliance $\Delta V/\Delta ICP$ (the ratio of a brain volume increase to the resulting pressure increase) is often low (Popp et al., 1995).

The proportion of extracellular and intracellular volume increases that occur in edema can be roughly determined by electron microscopy of tissue removed after surgery, which is rapidly fixed, or by perfusion-fixation of tissue *in situ* in animals (Van Harreveld, 1966; 1972; Bullock et al., 1991). Van Harreveld (1966) has pointed out that even perfusion-fixation can cause fixation-induced cell swelling due to cellular uptake of electrolytes, and he has shown that only rapid freeze substitution methods consistently gives reliable electron microscopy results.

An estimate of the size of the extracellular space has been made using electrical resistance (impedance) studies in experimental animals (Van Harreveld and Schade, 1962; Hossman, 1971; Hansen and Olsen, 1989) and it seems likely that such methodology can be applied clinically (Holder, 1992). The volume relations of the different intracranial compartments are as follows (Rapoport, 1979)

$$V_{Blood} + V_{CSF} + V_{Brain} = \text{constant} \quad (1)$$

$$V_{Brain} = V_E + V_I \quad (2)$$

where V_E = extracellular volume and V_I = intracellular volume. Relative changes in V_E and V_I can be measured with impedance methods utilizing equations originally derived by Maxwell, simplified to

$$\frac{1 - r_1/r}{2 + r_1/r} = \frac{\rho}{2} \quad (3)$$

where r_1 would represent the specific resistance of the extracellular space (V_E) and r the specific resistance of total brain ($V_E + V_I$); ρ = the fractional volume of the cells (Van Harreveld, 1966). This relation is based on the fact that the cells will essentially be nonconductors of current compared to the extracellular space. Alternating current is used to avoid polarization effects and neuronal excitation. At high frequencies, the capacitative component of the impedance will be much less than the resistive component. r_1 can reasonably be assumed to be the same as cerebrospinal fluid (50 Ωcm) and r can be measured. Values of 232 to 284 Ωcm have been measured for forebrain cortex (Van Harreveld, 1966). Substituting in equation 3 for a median value for r of 258, ρ = 0.73 giving an extracellular space

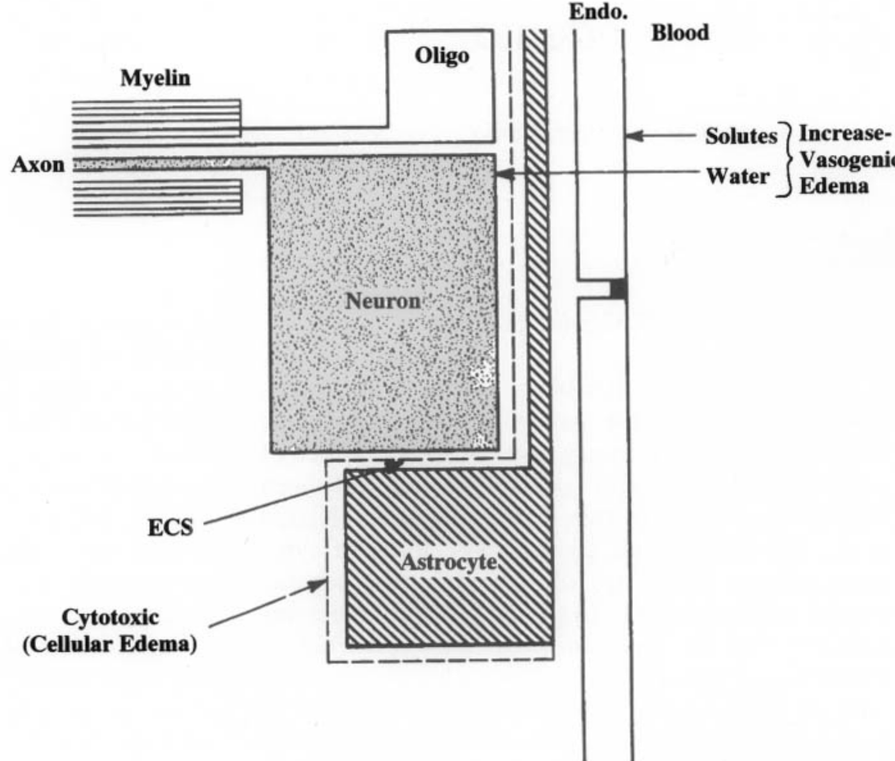


FIG. 61-1. Diagram of blood-brain barrier at the level of the brain capillary showing surrounding cells. Shown are the two different types of edema; vasogenic edema with net gain of solute and water due to loosening of the blood-brain barrier with entry of protein, salts and water. This is contrasted with cellular or cytotoxic edema principally involving intracellular swelling of astrocytes and dendrites (*not depicted*). See text for further discussion and contrast of cellular edema (gain of water) with cellular swelling (no gain of water) where cells, principally astrocytes, swell at the expense of the extracellular space. [From Kimelberg (1992), permission.]

of approximately 27%. Further refinements in techniques are needed to reduce errors, but in theory a dynamic readout of changes in the extracellular space as a function of stroke, epilepsy, and even neuronal activity are possible (Holder, 1992). It is also important that r_1 does not change due to alterations in its total electrolyte concentration. The principle of the lack of persistence of osmotic gradients for animal cells implies this will only occur if hypoosmolarity develops, and this can be measured if r_1 can be taken as the same as the specific resistance of the cerebrospinal fluid.

In vasogenic edema increased osmoles and water enter the brain generally due to breakdown of the blood-brain barrier. The blood-brain barrier is primarily due to intercellular occluding tight junctions between the endothelial cells lining the blood vessels and capillaries of the central nervous system of highly developed animals, and seems to be induced early on in development by factors, in part, produced by the surrounding astrocyte processes (Janzer and Raff, 1987; Neuhaus et al., 1991; Chan-Ling and Stone, 1992). The tight junctions exclude even small ions and only lipid-soluble substances are freely permeable (Davson, 1976; Goldstein and Betz, 1986; Abbott and Revest, 1991). Thus, very active transport systems are present for moving polar substances into and out of the brain. For example, the D-glucose transport system is present at a very high density, and there are a number of different amino acid carriers (Johansson, 1990). The existence of the blood-brain barrier means that a highly modified version of Starling's principle applies to the brain; excess water and solute are efficiently kept out, since any water entering the brain does so without solute and purely under hydrostatic pressure. The opposing osmotic force due to the retained ions and plasma proteins in the blood, then sets up an immediate opposing force driving the water back into the blood (Rapoport, 1979; Cserr and Patlak, 1991). With the breakdown of the blood-brain barrier, this control is lost, the barrier is breached and an ultrafiltrate of blood is driven by blood pressure into the brain. The blood cells are presumably retained by the residual permeability of the blood-brain barrier except in injury sufficient to lead to hemorrhage. Most of this ultrafiltrate fluid accumulates in the white matter due to the greater compliance of this region, that is, white matter will more easily accept the increased volume at a given pressure. This is likely due to the greater ease of separation of the parallel oriented white matter tracts (Rapoport, 1979).

The origin of cellular swelling is less clear and, as noted above, it has also been referred to as cytotoxic edema to distinguish it from vasogenic edema (Klatzo et al., 1984). It is very prominent in ischemia, hypoxia, and brain trauma, and under such conditions shows marked focal and dispersed swelling of the

soma and processes of astrocytes (Van Harreveld, 1972, 1982; Kimelberg and Ransom, 1986). Swelling of the astrocytic processes surrounding blood vessels and capillaries of the brain are very prominent in many pathological states (Table 61-1) and an illustration of perivascular astrocytic swelling after experimental traumatic brain injury is shown in Figure 61-2. Swelling of some neuronal elements, especially dendrites, is also seen, but swelling of the neuronal cell soma is more limited and swelling of oligodendroglia is generally absent (Van Harreveld, 1966; Van Harreveld and Fifkova, 1971). Reduction of the extracellular space is seen in ischemia as measured by an increase in brain impedance (Van Harreveld and Schade, 1962; Hossman, 1971; Van Harreveld, 1972; Hansen and Olsen 1989; and see above). Increased concentrations of membrane-impermeant molecules in the extracellular space are seen after irreversible ischemia, as measured by intracranial microdialysis, are also indicative of a decreased extracellular space (Katayama et al., 1992). However, in this model the decrease in extracellular space was transient occurring for only 2–3 minutes after ischemia. Also shown in this study is the rapid rise of $[K^+]_e$ (see Figure 61-3). However, actual concentrations in the extracellular space cannot be reliably obtained by microdialysis methods.

Figure 61-4 shows that in reversible cat brain ischemia cell swelling, assumed to occur because a decrease in the extracellular space was measured by an increased impedance (see Figure 61-4B), can precede actual edema formation as measured by a gain of brain water (Figure 61-4A). There was a rapid onset of cell swelling, but initially no net gain of brain water. However, large increases in $[K^+]_o$ would undoubtedly occur during this period (Hossmann et al., 1977; Katayama et al., 1992) (Figure 61-3), and this should activate astrocytic swelling as discussed below. However, since there is no gain of brain water during the ischemic period cell swelling could only be at the expense of a decreased extracellular space, measured by the impedance measurements. During the recirculation phase following the reversible ischemia a rapid increase in brain water occurs, presumably because water shifts from the now functioning vascular compartment to both the extracellular space and to the brain cells (see the first two paragraphs of this section). This gain of fluid could be due to a transient increased permeability of the blood-brain barrier or an increased osmolarity in the brain, such as increased breakdown of glycogen specifically localized in astrocytes (Kimelberg and Norenberg, 1994). Thus, based on the temporal relationships of Figure 61-4, the key events in setting the stage for edema formation (i.e., water gain) appear to have occurred early, during a phase of presumed pure cell swelling.

Clinically, astrocytic swelling has been described as a prominent and early feature associated with cerebral contusion in head-injured patients operated on for mass effects, as compared to brain tissue from patients operated on for epilepsy or gliomas. The tissue was removed and immediately immerse-fixed. As noted by Bullock et al. (1991): "Massive astrocytic swelling ("cytotoxic" edema) was seen 3 hours to 3 days after injury, maximal in perivascular foot processes, and compressing some of the underlying capillaries. The tight junctions were not disrupted. Neuronal damage was most marked three to 11 days after injury."

TABLE 61–1. *Different Pathologic States in Which Astrocytic Swelling Occurs*

	References
Ischemia	Garcia, 1984; Jenkins et al., 1982
Trauma	Nelson et al., 1982; Barron et al., 1988; Bullock et al., 1991; Bakay et al., 1977
Hepatic encephalopathy or Reye's syndrome	Norenberg, 1981; Davis et al., 1990
Hypoxia	Van Harreveld, 1966; Yu et al., 1972
Status epilepticus and hypoglycemia	Siesjo, 1981
Experimental allergic encephalomyelitis (EAE)	Eng et al., 1989
Chronic alcohol exposure (mice)	Smith and Davies, 1990

MECHANISMS OF CYTOTOXIC EDEMA

It remains obscure why astrocytic swelling occurs so frequently (Table 61-1). There have been numerous suggestions but little hard evidence due to the relatively limited work in this area and the difficulty of extrapolating work *in vitro* to properties *in vivo*. It is also undoubtedly a complex phenomenon *in vivo*, probably with several causes (Kimelberg, 1992). Early studies on astrocytic swelling had used brain slices as *in vitro* models (Bourke et al., 1983), but the relative contribution of neurons and glia to the overall swelling could not be distinguished in these studies although previous ultrastructural work had suggested a major contribution of astrocytes to the overall slice swelling. The most common current *in vitro* approach to studying pure astrocytic swelling

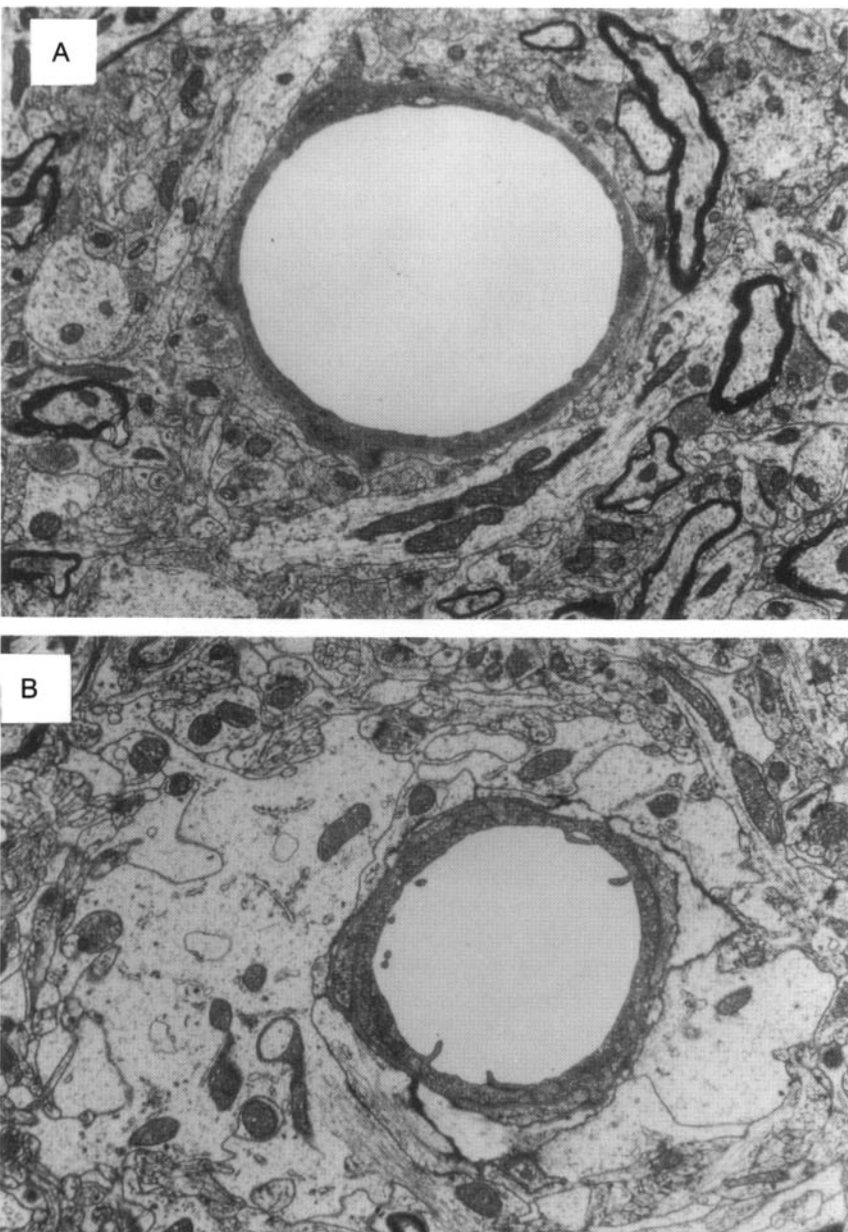


FIG. 61-2. (A) Electron micrographs of tissue from motor cortex of control, non-injured cats. (B) Electron micrograph of tissue from motor cortex of an experimental animal 40 minutes after closed head injury. Note swollen astrocytic profiles around capillary in bottom micrograph. [From Nelson et al. (1982), with permission.]

was first adopted in the early 1980s. It involved the use of primary monolayer cultures, that consist of 95% or more GFAP (+) astrocytes, prepared from neonatal rat brains. However, with such monolayer cultures there are problems in measuring cell volume in a dynamic and direct manner. In order to avoid the unknown changes in cell properties that could be caused by removing these anchorage-dependent monolayer cell cultures from the substratum on which they are growing radiolabeled intracellular space indicators have been used. Figure 61-5 shows such a study using [^{14}C]3-O-methyl-D-glucose, which is transported on the Na^+-independent, facilitatory diffusion glucose carrier present in these cells (also see below). It can be seen that in isotonic media the cells excluded [^{3}H] mannitol, used as an extracellular space marker, but when swollen by exposure to hypotonic media not only did the intracellular 3-O-methyl-D-glucose space increase, but [^{3}H]mannitol also appeared to enter the cell. Thus marked permeability changes appear to occur due to cell swelling that allow otherwise membrane-permeant molecules like mannitol to enter the cell.

Intracellular swelling can be due either to an uptake of osmoles such as Na^+, Cl^-, K^+ or glutamate (or some combination of these), from the extracellular to intracellular spaces with a shift of osmotically obligated water to the intracellular space

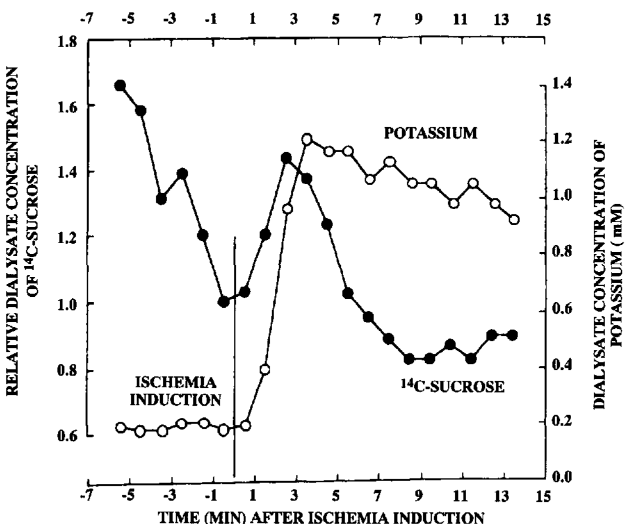
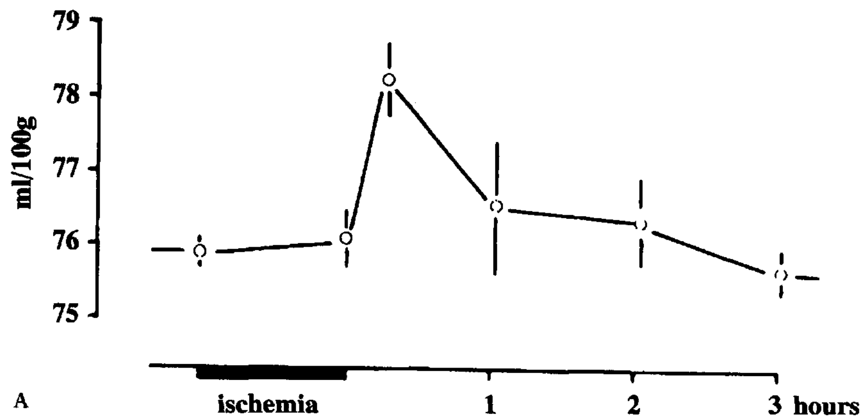


FIG. 61-3. Representative example of changes in extracellular K^+ (○) and ^{14}C-sucrose (•) measured from the same microdialysis probe after total irreversible cerebral ischemia in rats. [From Katayama et al. (1992), with permission.]

(Kimelberg, 1992), or generation of increased osmoles within the cell (so-called ideogenic osmoles). Figure 61-6 shows some of the major transport processes likely to be involved in cellular edema, superimposed on the model shown in Figure 61-1. In Figure 61-6, no. 1 shows influx of KCl due to Donnan forces driven by raised $[K^+]_o$ (Boyle and Conway, 1941; Barres et al., 1990a; Walz, 1992). Increased $[K^+]_o$ (>20 mM and up to 80 mM) is often seen in stroke and head injury (Kimelberg and Ransom, 1986; Siesjo, 1992; Nilsson et al., 1993). Direct measurements using ion specific electrodes in presumed glial cells (nonexcitable, large negative resting membrane potentials) in guinea-pig olfactory cortex have shown increased K^+_i and Cl_i^- activities with increased neuronal activity which was blocked by Ba^{2+} (Grafe and Ballanyi, 1987). Thus, this uptake is likely to be through K^+ channels, of which there are several types in astrocytes (Sonnhof, 1987; Barres et al., 1990a, 1990b). Cl^- channels will also be needed for KCl uptake. The Cl^- channels present in astrocytes are not usually open at normal resting membrane potentials, but a large conductance anion



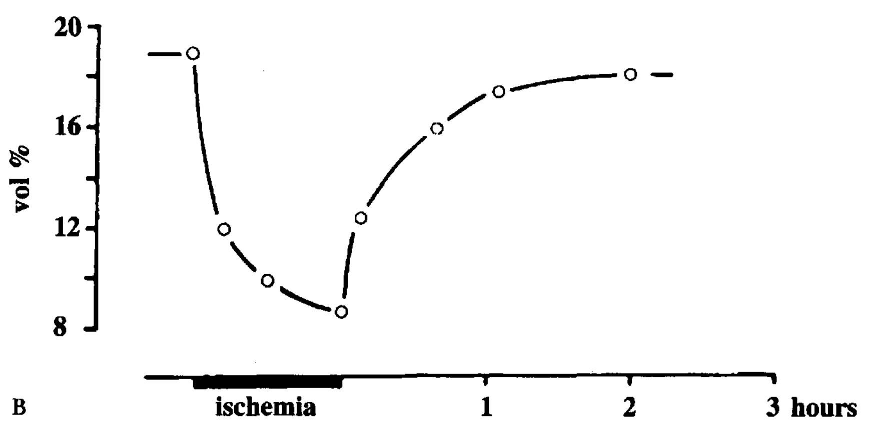


FIG. 61-4. Changes in (A) brain water and (B) extracellular space in cat brain during and after 1 hour of complete ischemia. Values are means ± SEM. Extracellular space was calculated from specific cortical impedance using the Maxwell equation (equation 3 in text). [From Hossman (1976), with permission.]

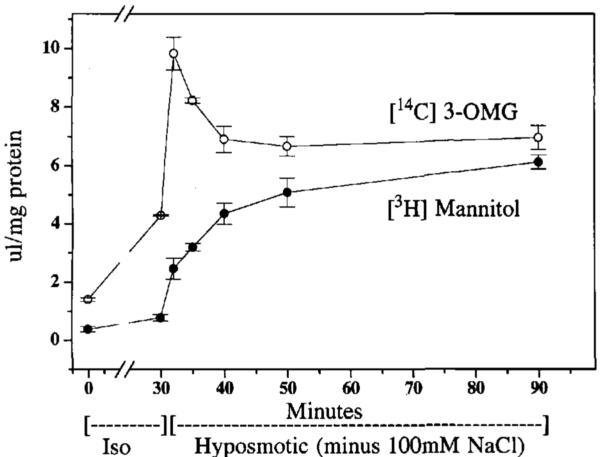


FIG. 61-5. Effect of exposing primary astrocyte cultures to hypotonic medium on the [^{14}C]3-OMG (○) and [^{3}H]mannitol (•) spaces. The first two values represent the zero time and the value after 30 minutes in isotonic medium, respectively. Thereafter, the cultures were exposed to hypotonic medium (100 mM NaCl removed) as indicated. Each point represents the mean ± SEM of four separate wells. [From Kimelberg et al. (1992), with permission.]

channel and the smaller conductance Cl^- channels so far seen in astrocytes are voltage-sensitive (Jalonen, 1993; Sonnhof, 1987).

Other mechanisms for astrocytic swelling that have been proposed are HCO_3^- and H^+ leaving the cell in exchange for Cl^- and Na^+, respectively on the appropriate exchangers (Figure 61-6, no. 2). Studies by Bourke et al. (1983) using brain slices and by Kimelberg et al. (1979) using primary astrocyte cultures, led to the suggestion that swelling may occur by simultaneous operation of Cl^-/HCO_3^- and Na^+/H^+ exchange transport, with H^+ and HCO_3^- cycling from the intra- to extracellular spaces via membrane-permeant CO_2. The HCO_3^- and H^+ ions can turn over many times by cycling into and out of the cell via membrane-permeant CO_2, with intracellular hydration to HCO_3^- and H^+ and extracellular dehydration back to CO_2 (Kimelberg et al., 1979; Kempski et al., 1990). The intracellular hydration of CO_2, and perhaps the extracellular too, is likely to be accelerated by carbonic anhydrase. Thus, there will be a net transfer of NaCl from the extra- to intracellular space. This NaCl is likely to be less effectively pumped out when the (Na^+,K^+) pump activity is reduced due to lowered intracellular ATP levels, and thus there will be intracellular swelling at the expense of the extracellular space. Swelling can also occur in ischemia because of the breakdown of macromolecular glycogen that is specifically localized in astrocytes (Kimelberg and Norenberg, 1994) to glucose, and finally to lactate, since there is a net generation of many moles of lactate from 1 mole of glycogen, which will lead to swelling of the intracellular compartment within which it is produced, if it is retained.

It has been shown that glutamate or kainic acid (KA) when injected into the central nervous system causes swelling of retinal Müller cells and astrocytes (Van Harreveld and Fifkova, 1971; Van Harreveld, 1982). These effects could operate through glutamate or kainic acid stimulating neurons and increasing production of the metabolic products CO_2 or H^+ leading to astrocytic swelling by the exchange processes described above, or glutamate or kainic acid could have a direct effect on astrocytes themselves. It is well established that astrocytes take up glutamate by a Na^+-dependent transport mechanism and such uptake could lead to swelling. However, [^{3}H]kainic acid is not taken up by primary astrocyte cultures (Kimelberg et al., 1989), and Sontheimer et al. (1988) showed that the depolarization of primary cortical astrocyte cultures by both L-glutamate and kainic acid is best described by the activation of an AMPA (aminohydroxymethylisoxazole propionic acid)/kainate ionotropic type of excitatory amino acid receptor. On this basis, swelling could be due to direct activation of astrocytic receptor channels leading to Na^+ entry. Cl^- would presumably enter on channels perhaps also activated by the AMPA/KA receptor or by membrane depolarization.

Fatty acids and free radicals can lead to swelling presumably because they can cause breakdown of the selective permeability of the membranes leading to nonselective ion influx. This influx should be principally of Na^+ and Cl^- because of the high extracellular concentrations of these ions. Because of the appearance of measurable free radicals and the protective effects of antioxidants, such as the 21 aminosteroids or "lazaroids" (Braughler and Hall, 1992), and superoxide dismutase (Kontos and Wei, 1986), free radicals are now considered to be an important component of damage during ischemia and trauma, and may be especially significant for long-term secondary effects. There are a number of free radical species which are oxygen-based, such as the superoxide anion $O_2\cdot^-$ (Kontos and Wei, 1986). These are produced because of incomplete reduction of oxygen by electron donors. Hydrogen peroxide (H_2O_2) can also be formed. The very short-lived hydroxyl free radical ($\cdot OH$) is formed from H_2O_2 in a reaction catalyzed by free iron. Such free iron can be liberated from the heme groups of hemoglobin or released from ferritin and transferrin after hemorrhage. These free radicals are deleterious due to their ability to form an oxidative cascade affecting the fatty acid side chains of membrane phospholipids, leading to peroxidatic

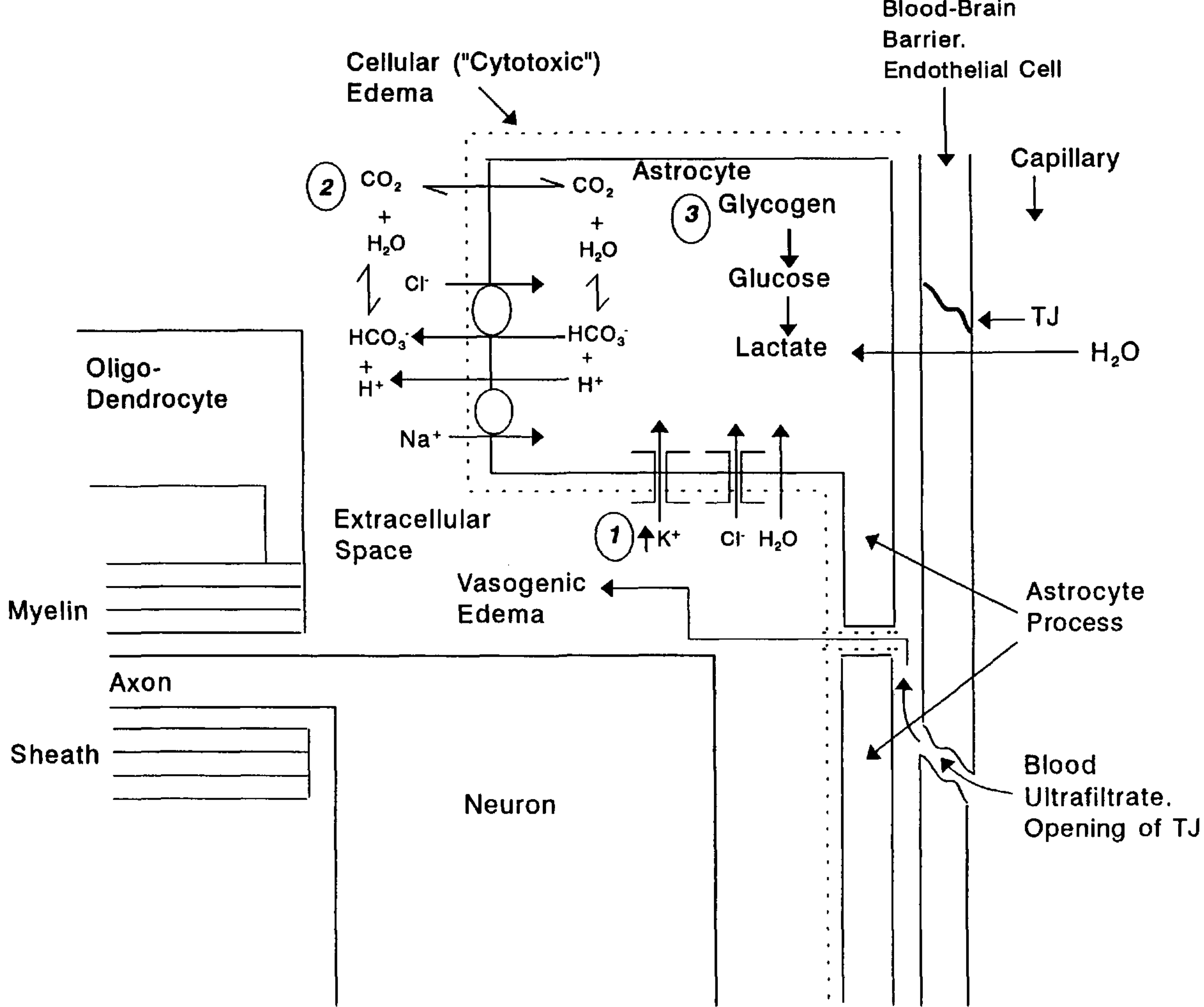


FIG. 61-6. Postulated mechanisms of cellular edema (also see text). For completeness vasogenic edema, due to "opening" of the tight junctions (*TJ*) between the endothelial cells with a blood ultrafiltrate moving into the brain driven by blood pressure, is also shown. Cellular or vasogenic edema can coexist but, in its purest form, cellular edema need not result in a gain of brain water because the cells can swell at the expense of the extracellular space. This can also occur under nonpathological intense neuronal activity and preferably should be referred to as "cellular swelling." Three postulated and numbered causes of astrocytic swelling are depicted in the diagram (see text for further details): 1, influx of K^+ and Cl^- driven by raised extracellular K^+ (Donnan swelling); 2, intracellular hydration of CO_2-producing H^+ and HCO_3^- and their exchange for extracellular Na^+ and Cl^-, respectively; 3, generation of osmoles within the cell, for example, by metabolism of the macromolecular glycogen in astrocytes to a number of moles of glucose and lactate. [From Popp et al. (1995), with permission.]

breakdown of the membrane phospholipids and the generation of further free radicals (Kontos, 1989). All these effects will break down the selective permeability of cell membranes leading to membrane depolarization and influx of Na^+ and Cl^- with water, and ultimately cell destruction. Free radicals can also directly affect proteins, such as glutamine synthetase (Halliwell and Gutteridge, 1985). Current debate centers on when and how these mechanisms might be involved in ischemia and trauma (Kontos, 1989). The effects of oxidation and free radicals on cells might be expected to be nonspecific and it is not clear why they would selectively produce swelling of astrocytes and dendrites. However, localized production of free radicals or increased susceptibility are a few of many possibilities. The free radicals might be expected to ultimately cause a breakdown of the blood-brain barrier, leading to vasogenic edema (Figure 61-6).

A number of transmitters or neuromodulators have been shown to produce HCO_3^--dependent swelling in brain slices (Bourke et al., 1983), and one of the most potent of these is adenosine (or the nonmetabolizable analogue 2-chloroadenosine). Adeno-

sine was found to lead to marked swelling of astrocytes as shown by electron microscopy after superfusion of adenosine over exposed cat cerebral cortex (Bourke et al., 1981). In brain slices *in vitro* adenosine may have direct effects on astroglial adenosine receptors or indirectly cause astrocytic swelling by activating neurons, while in intact animals adenosine also increases blood flow.

Increased $[Ca^{2+}]_i$ can be damaging to cells, and may also lead to swelling. Increased $[Ca^{2+}]_i$ will cause activation of Ca^{2+}-dependent proteases and phospholipases, leading to hydrolysis of proteins and membrane phospholipids, respectively. For example, phospholipase C breaks down membrane phospholipids into their constituent diacylglycerols and phosphoryl head groups. In pathological situations diacylglycerol can be further broken down by other phospholipases, namely A1 and A2, to liberate the two fatty acids bound to the glycerol backbone. The unsaturated fatty acids at position 2, such as arachidonic acid, are particularly pluripotential. The phospholipases, particularly the A forms, are very sensitive to Ca^{2+}. Free fatty acids lead to disruption of the lipid portion of the membrane by having a detergentlike effect, breaking apart the necessary hydrophobic packing needed to maintain the integrity of the lipid bilayer. This disruption will serve to short-circuit the normal impermeability of the lipid bilayer and thus the selective permeability of the cell membrane. These changes can also alter the lipid microenvironment of membrane proteins and consequently the activity of ion pumps and channels, also leading to dissipation of membrane gradients. Products of arachidonic acid, namely the leukotrienes and prostaglandins can also lead to activation of K^+, Cl^-, and Na^+ channels, which could lead either to cell swelling or shrinkage (Hoffmann and Kolb 1991).

Hepatic encephalopathy, as encountered in Reye's syndrome and other pathological states (see Table 61-1), has been shown to result in electron-lucent astrocytes, as seen by electron microscopy, indicating cell swelling. Such swelling could be related to metabolism of excess ammonia, formed in these disorders, by the glutamine synthetase that is present at high concentrations in astrocytes (Norenberg, 1981; Chapter 63, this volume). Another possibility is that increased ammonia enters the astrocyte across the blood-brain barrier to form ammonium ions intracellularly. Direct determinations *in vivo* with ion-specific pH microelectrodes have given a mean value for the intracellular pH of astrocytes of 7.04 with a range of 6.73 to 7.38 (Chesler and Kraig, 1989). This means that 99% of intracellular ammonia will be in the form of NH_4^+. An increase of NH_3, leading to a greater $[NH_4]_i$, would alkalinize the interior of the cell, leading to an increase in $[HCO_3^-]_i$, which could then exchange for $[Cl^-]_o$, resulting in an accumulation of NH_4Cl in the astrocyte. It is known that excess CO_2 exacerbates the effects of hepatic encephalopathy (Norenberg, 1981), and this could be a source of the increased intracellular H^+ and HCO_3^-.

Recently it has been reported that in the early stages of experimental allergic encephalitis (EAE) induced in rats by injection of purified guinea pig myelin, there is marked swelling of perivascular astrocytes associated with increased immunoreactivity for glial fibrillary acidic protein (GFAP) (see Table 61-1). This increased immunoreactivity occurs without an increase in total GFAP content, suggesting increased exposure of epitopes on GFAP, perhaps due to its depolymerization in swollen astrocytes (Eng et al., 1989).

Although it seems likely that astrocytes *in situ* mainly swell by mechanisms other than exposure to hypotonicity, significant hypoosmolarity is also seen clinically. Both hyponatremia, as well as hypernatremia, are encountered after traumatic brain injury (Popp et al., 1995), and hyponatremia due to inappropriate secretion of antidiuretic hormone is a common clinical problem. Swelling of primary astrocyte cultures or C6 glioma cells *in vitro* due to media made hypotonic by reduction of NaCl is followed by a characteristic regulatory volume decrease (RVD), and is associated with release of K^+, Cl^-, and amino acids (Kempski et al., 1983; Kimelberg and Frangakis, 1985; Olson et al., 1986; Pasantes-Morales and Schousboe, 1988; Kimelberg et al., 1990). Work *in vivo* has shown that astroglial swelling occurs and spontaneously resolves after experimental acceleration-deceleration closed-head injury (Barron et al., 1988). However, the time course of swelling and RVD *in vitro* after exposure to hypotonic media is faster than the onset of astroglial swelling and its resolution *in vivo*. This difference could be due to a balance of slowly developing swelling forces and normal RVD forces *in vivo*. However, this slower rate of swelling, is seen in primary astrocyte cultures using high extracellular K^+ or L-glutamate to swell the cells (O'Connor et al., 1993b). It is associated with a slower amino acid release (Kimelberg and Goderie, 1991) than the rapid initial release due to exposure to hypotonic media (O'Connor and Kimelberg 1993). This emphasizes the need for studying the kinetics of cell swelling to see how swelling *in vivo* compares to swelling *in vitro* due to different influences; in the next section experimental methods for measuring swelling are briefly discussed.

METHODS FOR MEASURING EDEMATOUS VOLUME CHANGES

Cell Monolayers In Vitro

A large amount of the work done on the mechanisms and consequences of cell swelling utilizes cell cultures for ease of analysis of volume changes in a homogeneous cell population. The use of cell cultures for *in vitro* studies on cell volume regulation has allowed researchers to focus on understanding mechanisms at the cellular level. Cell cultures can be classified according to a variety of criteria, including whether they are grown directly from tissue (primary cultures) or they are normal or transformed established cell lines. Primary cultures of normal neurons or glia are thought to be best for studying the properties of the normal central nervous system cell types.

Some cell types can be grown as suspension cultures, for example, lymphocytes, and such cells are very convenient for studies using the Coulter Counter or a light-scattering technique to measure cell volume. This Coulter counter technique has also been applied to C6 glioma cells (Kempski et al., 1983) and primary astrocyte cultures (Olson et al., 1986). Since these cultures grow as monolayers, the cells first have to be removed from culture dishes either by enzymatic treatment, usually with trypsin, by using a Ca^{2+} chelator or by mechanical scraping, to make a uniformly dispersed cell suspension. However, such detachment of substrate-attached cells will affect the shape and transport properties, including all aspects of passive, facilitated, and active permeability of cell membranes, introducing uncertainty with regard to the properties measured.

When cells are left in their normal attached state, intracellular volume has most frequently been measured using equilibration with [^{14}C−]3-O-methylglucose (3-OMG), as originally described for hepatocytes (Kletzien et al., 1975) and applied to primary astrocyte cultures by Kimelberg and Frangakis (1985) (see Figure 61-5). [^{14}C]urea has also been used in glial monolayer cultures (Martin and Shain, 1979). We have used both methods for primary astrocyte cultures, and, although both report regulatory volume decrease and regulatory volume increase response, such indirect radiolabeled tracer studies introduce methodological problems. One of the important drawbacks of this method is its inability to measure rapid changes in cell volume. This technique is limited to collecting data points at probably not less than 30-second intervals because one needs to completely remove the extracellular tracer by washing. Other problems concerning this technique are whether there is leakage of the tracer when the cell is swollen, what intracellular volume is actually being measured, that is, are the tracers excluded and/or concentrated in certain areas of the cell, does swelling and volume regulation cause the equilibrium rate of the tracer to change and are there impurities in the marker that would cause errors in the measurement of volume? It is also, of course, vital that the marker is nonmetabolizable as metabolism would cause serious errors in the volume measurement. For [^{14}C]3-OMG the cells must only have the equilibratory, phloretin-sensitive, Na^+-independent carrier, which has been shown to be the case in primary astrocyte cultures (Kimelberg and Frangakis, 1985), since 3-OMG is carried on the glucose carrier in a similar manner to glucose. However, in the presence of excess glucose any glucose gradient will cause an identical 3-OMG gradient to develop, which will affect the apparent intracellular 3-OMG space and therefore intracellular "volume."

As an alternative to the above methods an impedance method, similar to that used for *in vivo* measurements of extracellular space (see the section *Cellular and Vasogenic Edema*), has been developed for measuring dynamic changes in cell volume of primary astrocyte monolayer cultures (O'Connor et al., 1993b). In this method monolayer cell cultures are placed in a confined channel containing a salt solution and the impedance or electrical resistance of the small channel over the cells is measured using an applied AC field at 500 Hz, at which frequency the cell impedance is very high relative to the fluid impedance. If the volume of the cells increases, the volume of the solution within the channel available for resistive current conduction will decrease by the same amount. Figure 61-7 shows results using this method for three different effectors of cell colume changes, all added at 0 time. Exposure to hypotonic solutions shows the expected rapid initial swelling followed by a gradual regulatory volume decrease back to control volume. The slow rise in volume upon exposure to isosmotic medium containing increased K^+ (50 mM KCl replacing 50 mM NaCl), is consistent with a slow increase in volume due to KCl uptake. The slow rise response to 0.1 mM glutamate is also consistent with glutamate uptake. Thus this method allows measurements of volume changes in real time.

There are also a number of microscopic imaging methods, changes in fluorescence probe concentrations and laser and visible light scattering. These have been recently reviewed in detail elsewhere (MacKnight and Leader, 1989).

Volume Measurements In Situ

In order to correlate the findings in cultures or tissues *in vitro* with behavior of cells *in vivo* it is nec-

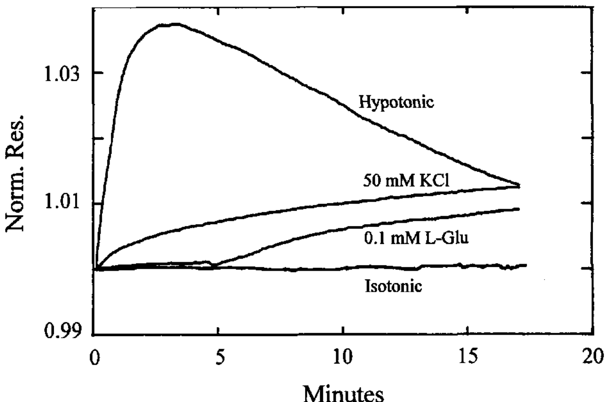


FIG. 61-7. Volume increases in primary astrocyte cultures, measured as increases in normalized resistance (y axis) using an impedance method. These were produced by exposure to hypoosmotic media (minus 100 mM mannitol), isoosmotic 50 mM KCl media (replaces equivalent NaCl) and 0.1 mM glutamate as indicated, all starting at $t = 0$. An isotonic control exposure is also shown (*bottom trace*). See text for further details. [From data in O'Connor et al. (1993b) with permission.]

essary, of course, to have methods for making such measurements. MacKnight and Leader (1989) have recently discussed such techniques in detail for epithelial cells. Ransom et al. (1985) used a membrane impermeant cation TMA^+ (trimethylammonium) injected into the extracellular space and measured its activity with potassium selective electrodes, which are far more sensitive to TMA^+ than K^+. As the nerve was stimulated extracellular TMA^+ activity rose, consistent with a decrease in the extracellular space. Also this decrease was proportional to changes in extracellular potassium, which causes swelling of glial cells. Developmental aspects of the study also indicated that it was likely to be the glial cells that were swelling. Ballanyi et al. (1990) have also used TMA^+, or choline, which behaves similarly, and recorded intracellularly from leech neuropil glial cells which had accumulated these compounds after bath application. It was shown that elevation of the extracellular potassium concentration caused a depolarization of the cell membrane potential as expected and a decrease in the intracellular choline signal, consistent with the neuropil leech glial cells having swollen.

As discussed in detail in the first section of this chapter, impedance methods were applied by Van Harreveld (1966) and Hossman (1971), to mammalian brain *in vivo*. These methods allow only measurements of the overall extracellular/intracellular changes. In these pioneering studies it was shown that conditions such as asphyxia or ischemia caused a marked increase in the measured impedance of the tissue consistent with cellular swelling and reduction of the extracellular space. An example of the impedance method applied to global cerebral ischemia in the cat was shown in Figure 61-4B. During complete ischemia there was an initial 50% decrease in extracellular space, presumably due to rapid onset of cell swelling, but no net gain of brain water (Hossman et al., 1977). During the ischemic phase, large increases in $[K^+]_o$, due to loss of K^+ from and gain of Na^+ by cells (see Figure 61-3) would undoubtedly have occurred (Hossman et al., 1985), and should activate astrocytic swelling. However, since there was no gain of brain water, this swelling could only be at the expense of a decreased extracellular space, consistent with reuptake of KCl, with the cells retaining the Na_i^+ already gained. Other factors such as an increase in intracellular osmolarity should also occur because of increased glycogenolysis and lactate production. During the "recirculation phase," a rapid increase in brain water occurred (Figure 61-4A), presumably because water shifts from the recirculating blood to both the extracellular space and brain cells. This fluid shift could be due not only to an increased osmolarity of the brain, but also a transient increased permeability of the blood-brain barrier. However, this was not shown. Since ischemia has been shown by electromicroscopy in many experiments to lead preferentially to astrocytic swelling (Jenkins et al., 1982; Garcia, 1984; Kimelberg and Ransom, 1986) it is quite possible that a major contribution to the decrease in the extracellular space seen in Figure 61-4B was astrocytic swelling. However, Van Harreveld and Fifkova (1971) also found, using rapid freeze substitution techniques with electron microscopy, that under the same conditions there was marked dendritic swelling. The relative contributions of glial and dendritic swelling to the decreased extracellular space seen in ischemia cannot be determined using the impedance methodology alone.

Note Added in Proof: Magnetic resonance imaging (MRI) especially a technique termed *Diffusion Weighted* (DW) MRI, is currently being used to measure movement of water from the extra- to intracellular space (Campagne et al., 1994).

CONSEQUENCES OF ASTROCYTIC EDEMA

Regulatory Volume Decrease and Release Phenomena

Primary astrocyte cultures, when swollen in hypotonic media, show RVD as do most cells, reestablishing their preswelling volume by losing ions (Kimelberg and Frangakis, 1985; O'Connor and

Kimelberg, 1993a). They also release amino acids such as [^{3}H]taurine, [^{3}H]D-aspartate and [^{3}H]L-glutamate, which these cells had previously accumulated, or the endogenous amino acids (Pasantes-Morales and Schousboe, 1988; Kimelberg et al., 1990; Pasantes-Morales et al., 1990). *In vivo*, significant hypoosmolarity is almost always due to lowered plasma Na^+ concentration as in hyponatremia, and this condition also leads to loss of taurine, aspartic and glutamic acids from the brain (Gullans and Verbalis, 1993). In Figure 61-8 we also show release of [^{3}H]D-aspartate from primary astrocyte cultures due to exposure to high K^+ media. This release has a slower onset time than hypotonic media-induced release, but is also inhibited by L-644,711 (see next paragraph).

Clinically hyponatremia, and also hypernatremia, are encountered as one of the systematic manifestations of traumatic brain injury (Popp et al., 1995). The finding of marked release of glutamate by swollen astrocytes leads to the interesting possibility that, when astrocytes swell *in vivo*, they release excitotoxic amino acids such as L-glutamate and L-aspartate. This in turn can cause neuronal injury due to excessive activation of NMDA and other glutamate receptors, as postulated in the excitotoxicity concept (Choi, 1988). Thus any drugs that are found to inhibit this release, as astrocytes are a potential source of L-glutamate released in pathological states (Farinelli and Nicklas, 1992), may be effective in alleviating secondary injury in stroke or trauma. This hypothesis is supported by the protective effects of the anion transport blocker L-644,711 in animal models of closed head injury and focal ischemia (Cragoe et al., 1986; Kimelberg et al., 1987; Kohut et al., 1992). *In vitro*, L-644,711 and other anion transport inhibitors including SITS, inhibit swelling-activated release of [^{3}H]taurine and [^{3}H]L-glutamate from swollen astrocytes (Kimelberg et al., 1990) (Figure 61-8). However, these anion transport inhibitors have not been investigated extensively in relation to other possible ways they can affect ischemic or other damage so that other reasons for the protective effects of L-644,711 are possible. Thus, L-644,711 has been shown to inhibit superoxide release from neutrophils (Bednar et al., 1992). However, L-644,711 does not act as a direct antioxidant of lipid peroxidation (Metsä-Ketelä, University of Tampere, unpublished observations).

How Is Astrocytic Swelling Transduced to Release Phenomena?

Role of Calcium. Because of the potential importance of astrocytic swelling to neuronal damage, mechanisms of volume regulation and the consequences of astrocytic swelling are now being studied more fully. Both extra- and intracellular Ca^{2+} have been shown to influence cellular volume regulation in a number of different cell types (McCarty and O'Neil, 1992). In some cells, removal of extracellular Ca^{2+} prevents osmolyte efflux and blocks volume recovery. In the case of primary astrocyte cultures, both extracellular and intracellular Ca^{2+} have also been found to be required for volume regulation as measured by the impedance method (O'Connor and Kimelberg, 1993a). Bender et al. (1992) have shown that, using radiolabeled 3-O-methyl-D-glucose to measure changes in cell volume, calmodulin antagonists completely inhibit RVD in primary astrocyte cultures.

Most vertebrate cells accomplish RVD from hyposomotic swelling principally through efflux of K^+ and Cl^- (McCarty and O'Neil, 1992). In some cells

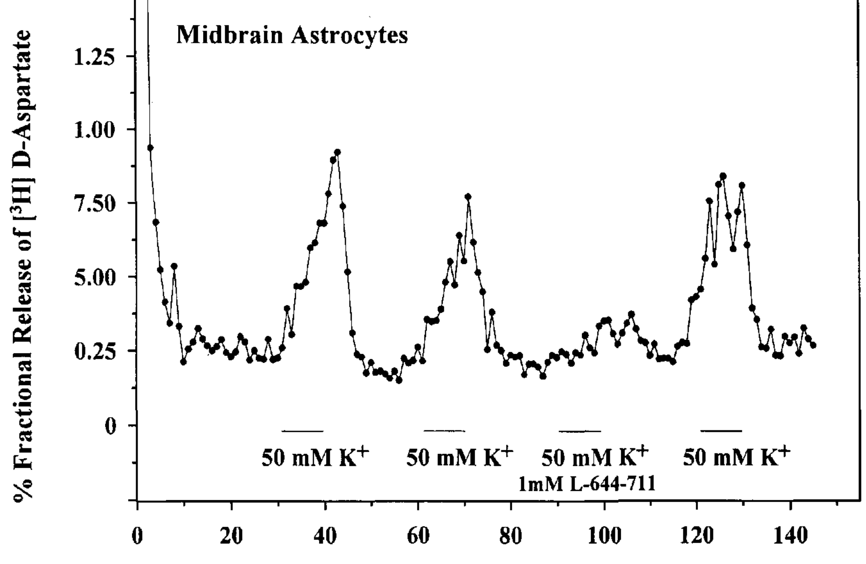


FIG. 61-8. Release of previously accumulated [^{3}H]D-aspartate from astrocytes prepared from the midbrain region of 1-day-old Sprague Dawley rats and grown for 4 weeks. Release was measured at 37°C, using a constant perfusion system, in which the control HEPES solution was changed, as indicated, to media containing 50 mM K^+ by isotonic replacement of Na^+ with K^+, plus and minus the anion transport inhibitor L-644,711. Results are expressed as a percentage of the radioactivity present at the beginning of each 1-minute collection period obtained by sequentially adding back the radioactivity released. [From Kimelberg and Goderie (1991), with permission.]

this is through separate K^+ and Cl^- channels (Hoffmann and Kolb, 1991), while in others the K^+ movement is totally Cl^--dependent and may be due to KCl cotransport (McCarty and O'Neil, 1992). A swelling-induced rise in Ca^{2+} often seems to stimulate K^+ movement through channels in RVD because both $[Ca^{2+}]_i$ rises and quinine and other compounds that inhibit Ca^{2+}-dependent K^+ channels, block or reduce cell volume recovery, for example, in Ehrlich ascites cells (Hoffmann and Kolb, 1991). However, recent data have shown that quinine can also inhibit anion channels (Roy and Malo, 1992). In primary astrocyte cultures Ca^{2+} seems to be required for swelling-induced K^+ and Cl^- efflux, and quinine also blocks RVD in these cells (O'Connor and Kimelberg, 1993a). Ca^{2+}-activated K^+ channels have been reported in astrocytes (Barres et al., 1990a; Tse et al., 1992) and in retinal Müller cells (Puro et al., 1989). One possible source of the increase in free Ca^{2+} could be increased Ca^{2+} influx into the cell through voltage-dependent Ca^{2+} channels or through swelling-activated nonspecific cation channels that are permeable to Ca^{2+}. An additional source is release from intracellular Ca^{2+} stores. Hypotonic media-induced swelling of astrocytes has been shown to cause a significant increased uptake of extracellular Ca^{2+} as measured with radiotracer $^{45}Ca^{2+}$ added extracellularly, and this uptake was partially blocked (60%) by the Ca^{2+} L-channel blocker nimodipine at 10^{-6}M. Increased $[Ca^{2+}]_i$ released from internal stores due to hypotonic swelling was also shown in the same study using fura-2 (O'Connor and Kimelberg, 1993a). This uptake into astrocytes upon swelling may be, in part, responsible for the marked decrease in extracellular Ca^{2+} seen in spreading depression, ischemia and trauma when astrocytes are also swollen (Hansen, 1985; Nilsson et al., 1993).

Other Second Messengers. In contrast to the amount of information on Ca^{2+} and volume regulation, there is a relative paucity of information regarding the involvement of other second messengers in cell volume regulation. Watson (1989) showed an increase in both intracellular calcium and cAMP subsequent to hypotonic media-induced swelling in mouse lymphoma cells. In a later study, however, he indicated that the cAMP increase did not appear to be involved in either the initial volume expansion or the extent of the subsequent regulatory volume decrease (Watson, 1990). Meijer and Hue (1991) have shown that the swelling of hepatocytes increases intracellular levels of inositol 1,4,5-triphosphate (IP_3), Ca^{2+} and cAMP. Haussinger and Lang (1991) have emphasized that cell swelling can accompany activation of second messengers by various hormones and may be a component of metabolic control in liver and other cells. There is clearly a need to study further the activation of IP_3 and cAMP, as well as Ca^{2+}, in astrocytes due to swelling, and the physiological consequences of such changes. Bender et al. (1993) have recently reported increased phosphatidylinositol hydrolysis in primary astrocyte cultures exposed to hypoosmotic media. This increased hydrolysis was blocked by a phospholipase C inhibitor, which also inhibited regulatory volume decrease.

Potassium and Anion Channels Activated in Astrocytes by Swelling

K^+ (measured with $^{86}Rb^+$) and $^{36}Cl^-$ efflux studies have shown swelling-induced release of K^+ and Cl^- from astrocytes *in vitro* (O'Connor and Kimelberg, 1993b). Swelling-activated membrane potential changes and whole-cell currents have also been reported in the same types of cultures (Kimelberg and O'Connor, 1988; Kimelberg and Kettenmann, 1990). These data strongly suggest that changes in channel activity occur during regulatory volume decrease in astrocytes *in vitro*. One of the first functions supporting the survival of single-cell organisms would have been the ability to regulate their cell volume in an environment of changing osmolarity, and also cell swelling due to Donnan forces because of impermeant intracellular macromolecules. Thus, swelling-induced channels may represent a well-conserved channel family. Mechanical stress, stretch, bending and shearing of the cell membrane are postulated direct signals for the activation of such channels. Patch-clamp techniques have given the possibility of mimicking these mechanical forces by applying pressure directly to the cell membrane and then recording changes in ion channel activity in the same patch. Such perturbations are found to activate channels, which were originally described in cultured muscle cells and termed stretch-activated ion channels or SACs (Guhary and Sachs, 1984). SACs have now been found in a wide variety of cell types ranging from bacteria to mammalian cells (Kullberg, 1987; Sackin, 1994).

The channels opened by the microelectrode pressure technique in animal cells are usually nonspecific cation channels and are permeant to divalent cations such as Ca^{2+}, as well as K^+ and Na^+ (Guhary and Sachs, 1984; Kullberg, 1987; Sackin, 1994). K^+ and nonspecific cation SACs, measured in the cell-attached mode with pressure applied through the cell-attached patch pipette, have been found at high densities in primary astrocyte cultures. In this comprehensive study both nonspecific cation and K^+-

selective channels sensitive to increased, or both increased and decreased pipette pressures were found (Bowman et al., 1992).

A number of recent studies have now shown anion currents and anion channels that are activated by swelling and/or pipette suction. One type is a large conductance anion channel of around 300 pS, which is seen in a number of cell types. Other anion channels of 7 pS and 20 to 30 pS conductances, respectively, have also been reported (Sackin, 1994). It is possible that some of these channels also carry amino acids, albeit with lower conductances (Roy and Malo, 1992). As noted in the section *Regulatory Volume Decrease and Release Phenomena* astrocytes *in vitro* release amino acids when swollen, and this may occur through such channels. In the limited extracellular space of the brain such release of glutamate and aspartate is potentially neurotoxic (Choi, 1988). However, while swelling-induced $^{36}Cl^-$ efflux in primary astrocyte cultures is Ca^{2+}-dependent, amino acid efflux appears to be Ca^{2+}-independent (O'Connor and Kimelberg, 1993). Thus we may need to postulate different channels for Cl^- and amino acids in astrocytes. Studies on astrocytes in the cell-attached mode have shown that hypotonic recording solutions, in the cell-attached mode, increases the occurrence of a high-conductance (250–300 pS), voltage-sensitive Cl^- channel in cultured astrocytes (Jalonen, 1993). This same channel is frequently seen in inside-out excised patches in cultured astrocytes (Gray and Ritchie, 1986; Sonnhof, 1987; Jalonen, 1993), and in acutely isolated GFAP (+) astrocytes (Jalonen et al., 1992). It is inhibited by the anion transport inhibitor L644,711 (Jalonen, 1993) which also inhibits swelling-induced efflux of amino acids from astrocytes (Kimelberg et al., 1990), supporting the possibility that glutamate and aspartate can also pass through this channel. Used *in vivo* L-644,711 reduces injury in animal models of both head injury and stroke (see next section). For the large anion channel studied in astrocytes, the delayed activation seen after excision of the patch suggests that this channel is normally always closed in the resting state and becomes activated when the cell swells (Jalonen, 1993).

POTENTIAL THERAPIES OF BRAIN EDEMA

Treatment of brain edema where there is a net gain of fluid is naturally directed at reducing the brain swelling. Immediate benefit can be achieved by lowering P_{CO_2} by hyperventilation; this reduces cerebral blood volume, with a consequent rapid reduction in intracranial pressure (Popp et al., 1995). Another common approach is the use of intravenous mannitol to make the blood hyperosmotic to withdraw water from the brain (Pollay, 1985). However, mannitol will only work in this way where the blood-brain barrier remains intact, and there often appears to be a delayed rebound phenomena due to mannitol entering the brain. *In vitro*, mannitol also enters swollen astrocytes (see Figure 61-4).

Other therapies are targeted to likely effectors or secondary consequences of edema. Such therapies include glutamate receptor antagonists, Ca^{2+} transport blockers and free radical scavengers of antioxidants (McIntosh, 1993). The way in which cell swelling can lead to some of these consequences has been discussed in previous sections. Therapeutic strategies have also been directed to inhibiting astrocytic swelling. In an experimental (cat) closed-head injury model, a significant decrease in mortality and improvement in the rate of neurological recovery was found with L-644,711 which also inhibited astrocytic swelling in this same model (Nelson et al., 1982; Kimelberg et al., 1987; Barron et al., 1988), presumably by blocking anion channels or other anion transport systems. The compound was given after the initial trauma, but before a secondary imposed hypoxic period. L-644,711 has also been found to be effective in a rabbit model of focal ischmia where it reduces the infarct size by up to 85% (Kohut et al., 1992) and has been reported to cause remarkable recovery from an otherwise lethal tyramine-induced model of Reye's syndrome in dogs, which shows brain edema (Faraj et al., 1988).

Mechanosensitive channels and mechanosensitive secondary messenger systems are attractive candidates for a link between the immediate mechanical forces set up by head injury and the harmful secondary events, including brain edema, which are thought to be highly significant in determining the outcome from a traumatic brain injury (TBI) that is not immediately fatal (Popp et al., 1995). The unique component of TBI are the short-lived and random forces acting on the brain due to the acceleration/deceleration forces experienced by brain tissue caused by the sudden blows sustained in TBI. This "commotio cerebri" sets up shear forces within the brain tissue, and also bruising due to impact of the brain on the internal surfaces of the skull. The shear forces are thought to be the cause of the diffuse axonal injury seen in TBI, where axons are disrupted at right angles to their longitudinal axes, but even these effects may be delayed and thus are likely to be secondary to the mechanical forces (Povlishock, 1985). Thus influx of Ca^{2+}, Na^+ (with Cl^-), on mechanosensitive channels leading to swelling and all the processes dependent on the adenyl cyclase and

phosphatidylinositol-linked second-messenger systems may be directly and rapidly activated by the forces encountered in TBI, acting at the cellular level. Influx of Ca^{2+} can then lead to transmitter release and K^+ leading, in part, to the astrocytic swelling and secondary edema occurring in TBI. Such swelling will be further exacerbated by ischemia and hypoxia, which are secondary events in TBI, but could also be a result of a primary activation of mechanosensitive events causing vasoconstriction due to influx of Ca^{2+} into arteriolar smooth muscle or release of vasoconstrictors. Although purely speculative, mechanosensitive channels and other mechanosensitive processes could be a link between mechanical trauma and subsequent metabolic changes and thus be useful targets for future therapies.

CONCLUSIONS

The characteristics of brain edema can range from a pure cell swelling with no observable gain of water, to a gain of water with increased intracranial pressure with or without cell swelling. Cell swelling, principally of astrocytes, probably occurs under normal conditions in a controlled fashion where it may be a concomitant of ion homeostatic processes in the astrocytes, and as such can occur during periods of intense neuronal activity where no pathology occurs (Ransom et al., 1985). However, in a pathological state there is also energy failure and such swelling can be of a sufficiently long duration to be pathological, for any of the reasons discussed in the preceding sections.

Studies related to cell swelling *in vitro* often involve studies on astrocytes, since these, as shown by electromicroscopy, are the major cells that appear swollen in pathological states such as trauma and ischemia. However, this should not be taken to exclude the contribution of other types of cells swelling in cellular edema, and indeed marked dendritic and some axonal swelling was observed in the early electron microscopic work of Van Harreveld (1966).

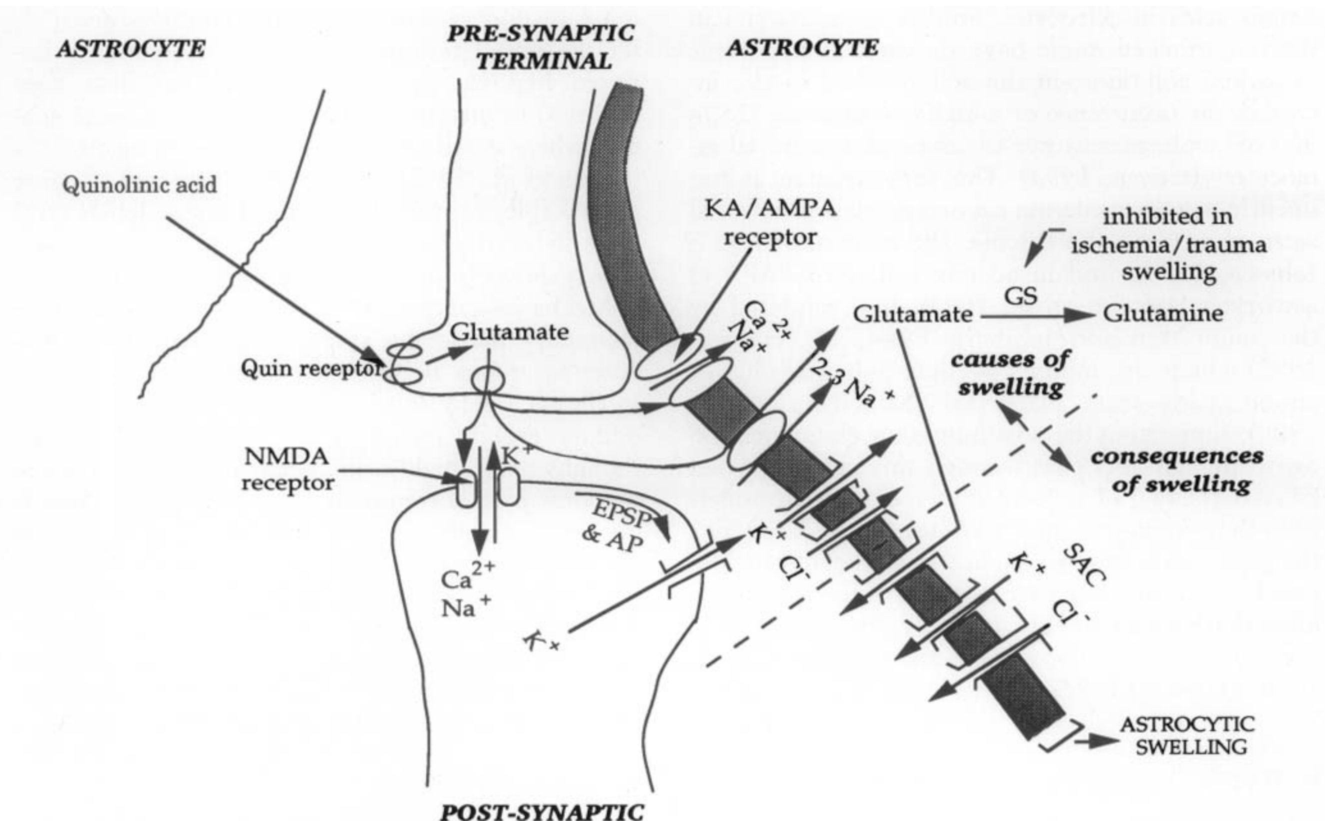


FIG. 61-9. Possible causes and effects of astrocytic swelling on glutamate and K^+ uptake and release by astrocytes. Causes of astrocytic swelling (*upper left of dotted line*) include activation of the astrocytic KA/AMPA receptor by glutamate leading to influx of Na^+ and Ca^{2+}, uptake of excess glutamate with Na^+ and uptake of KCl due to increased $[K^+]_o$ (Donnan uptake). On the *lower, right half of the dashed line* are shown possible consequences of astrocytic swelling. These include increased release of glutamate or KCl, thus short-circuiting these normally protective uptake mechanisms. In addition, the inhibition of glutamine synthetase (*GS*) reported to occur in ischemia which will lead to increased glutamate concentrations in astrocytes is also shown. *SAC*, stretch activated channels.

These other possible cell contributions have yet to be studied in detail. Studies *in vitro* have indicated that astrocytes can swell due to uptake of KCl or glutamate. When exposed to hypotonic solutions astrocytes swell rapidly and then regulate their volume back to normal, that is, they show regulatory volume decrease due to changes in their membrane permeabilities. These include increased permeability to Ca^{2+}, K^+, Na^+, and Cl^-. The release of K^+ and Cl^- occurring after swelling is a major component of regulatory volume decrease. Transport of these ions into and out of astrocytes can contribute to the changes in extracellular ion levels seen in pathological states *in vivo*, namely the marked falls in extracellular Ca^{2+} and Na^+ and increased K^+, seen in ischemia and spreading depression. The increased $[K^+]_o$ will, in turn, lead to cell swelling and thus to the decreased extracellular space measured *in vivo*. Possible deleterious consequences of cellular edema involving astrocytic swelling, include the release of neuroactive agents such as excitatory amino acids, changes in extracellular ion levels and a decreased extracellular space. Possible mechanisms for the swelling-activated release processes have been discussed in this chapter. All these effects are depicted in the speculative scheme shown in Figure 61-9. Successful future therapies for cellular edema occurring in cerebral ischemia and trauma are likely to be more fruitfully addressed when the mechanisms of swelling are better understood, and the benefits of such therapies will be better appreciated when the consequences of astrocytic and other cell swelling are more precisely defined.

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62 Epilepsy

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Epilepsy is a condition in the human or animal brain where—unrelated to an acute infection or metabolic disorder—seizures occur repetitively. A seizure is a momentary disturbance of neuronal function associated with motor behavioral abnormalities and disturbances of attention, cognition, and consciousness. Seizures are always caused by a strong synchronization of neuronal activity in a large population of central neurons. The seizures may or may not be associated with large increases in average firing rates (Ayala et al., 1973; Kostopoulos et al., 1983). Seizures with strongly increased firing rates account for the typical tonic and clonic contractions of body musculature, provided the underlying activity has recruited the motor system.

Attacks of hyperactivity are not restricted to epilepsy but occur also in neuralgia and causalgias. These are pain syndromes that seem to depend on repetitive firing in nerve fibers. These pain attacks are like convulsant seizures, sensitive to drugs that suppress repetitive firing (e.g., carbamazepine, phenytoin) (Macdonald et al., 1986). Focal or primarily generalized convulsant seizures are, in addition, sensitive to gamma-aminobutyric acid (GABA) mimetic drugs such as the benzodiazepines and the barbiturates (Macdonald et al., 1986).

Status epilepticus is a condition in which seizures recur frequently. In this sense, most acutely induced seizure models are conditions of a status epilepticus. The convulsant status epilepticus is a life-threatening condition due to development of brain edema (Dietzel et al., 1986), changes in blood pH and ionic alterations in the blood plasma, disturbances in circulation and respiratory control (drop of blood pressure), and hormonal regulation. Status epilepticus combined with hypoxia is a condition in which neuronal damage is easily provoked (Meldrum et al., 1993). The convulsant status epilepticus, if not blocked immediately, can become, after some time of ongoing activity, refractory to presently available anticonvulsant drugs, including the barbiturates and benzodiazepines as well as valproic acid.

Usually, seizures can be controlled by anticonvulsant and antiabsence drugs. However, between 10 and 20 percent of epileptic patients do not experience complete control of their seizure disorder by presently marketed drugs. Most drug-resistant seizures are generated in the temporal lobe. These seizures commence most frequently in the hippocampus, but sometimes also in the entorhinal cortex (Wilson et al., 1991). Temporal lobe epilepsy is associated with a reduction of nerve cell numbers in the hippocampal formation, including the entorhinal cortex, the hilus of the dentate gyrus, the cornu ammonis, and the subiculum. The tissue is characterized also by a proliferation of glial cells (hippocampal sclerosis) (Sloviter, 1983).

The processes that render the brain prone to the generation of seizures are often unknown. Disturbances in cell migration and maturation of neurons, induction of malformations such as microgyri, double cortex with a preserved subcortical plate, and survival of normally transient cells such as the horizontal cells in the neocortex and hippocampus, are among the developmental abnormalities involved in epileptogenesis (Wolf et al., 1993). Increased frequencies of surviving horizontal cells are also found in genetically determined epilepsies such as the absences (Meencke et al., 1984; Meencke, 1987). Lesion-induced alterations in the central nervous system often also underlie epileptogenesis. These include trauma, infection, and hypoxia- or ischemia-induced lesions, as well as to changes observed after some forms of status epilepticus (Kapur et al., 1989). Tumors are also often the cause of seizures. In a great number of epilepsies even a detailed morphological analysis does not reveal obvious causes of seizures (Wolf et al., 1993). These are therefore most likely dependent on functional alterations in nerve cells and glia.

Glial cells may well be involved both in epileptogenesis as well as in ictogenesis. Focal or global alterations in radial glia may be critical for migration and maturation disturbances (McConnell, 1991; O'Rourke et al., 1992; Roberts et al., 1993). Microglial activation causes neurodegenerative alterations with stripping of dendritic contacts (Gehrmann et al., 1991) and immunological reactions that may damage the neurons. They also could be involved in the formation of reactive astroglia. Factors released

from astroglia and microglia might affect the neuronal behavior and precipitate seizures, for example, due to blockade of potassium and activation of calcium currents (Plata-Salamán et al., 1992; Relton et al., 1992; Plata-Salamán et al., 1993). Alterations in oligodendroglia may be involved in the generation of action potentials in ectopic sites comparable to neuralgias. Disturbances in glial cell metabolism might affect the synthesis of the inhibitory neurotransmitter GABA. Glial changes in excitatory amino acid transport may be involved in the generation of seizures (Barbour et al., 1989; Flott et al., 1991; Piani et al., 1993). Changes in spatial potassium buffering as well as alterations in K^+ homeostasis may contribute to the generation and spread of seizures (Grisar, 1986; Yaari et al., 1986). Release of growth factors could affect sprouting, increase excitatory coupling, and thereby contribute to the formation of an epileptogenic zone.

Epilepsy as a condition for augmented readiness to generate seizures is often not stable. As a rule of thumb, 30% of epilepsies heal, 30% remain stable, and 30% become worse. This may involve the transition from nonconvulsant seizures, such as petit mal seizures, to convulsant primarily generalized or partial seizures with secondary generalization or the transition from drug-responsive to drug-resistant seizures. None of the presently available drugs prevents the progression from drug-sensitive to drug-resistant seizures nor the development of seizures following brain lesions. A reasonable percentage of these patients is treated with some success by surgery. This provides us with a good source of tissue in which alterations of neuronal and glial function, possibly leading to seizures or providing for an augmented readiness to generate seizures, can be studied.

MECHANISMS UNDERLYING SEIZURE GENERATION

Seizure generation (ictogenesis) and epileptogenesis are two different processes. Epileptogenesis is the process underlying a change in neuronal and glial cells, as well as in network properties, which facilitates the generation of seizures. Ictogenesis is the actual process of generation of relative short disturbances of neuronal function, in which many nerve cells are synchronously active. These states are often associated with neuronal "hyperactivity." Primarily generalized seizures are considered as different from focal and eventually secondarily generalized seizures. Primarily synchronized seizures are fits that display a simultaneous onset of seizure activity in electroencephalographic (EEG) recordings from both hemispheres. They can be of convulsant and nonconvulsant nature. Focal seizures commence in a circumscribed area and then eventually recruit more and more of the central nervous system into seizure activity.

A seizure is likely to occur when the balance between excitation and inhibition is disturbed. This balance is often intact between seizures. The balance between excitation and inhibition is organized on two levels: the synaptic and the cell intrinsic levels. On the synaptic level, either disturbance of GABA function or augmentation of glutaminergic function are essential. Thus a reduced production of GABA (Meldrum, 1989), diminished excitability of GABA-ergic cells (Bekenstein et al., 1993), diminished GABA release, alterations in postsynaptic receptors (Kamphuis et al., 1989; Kamphuis et al., 1991), disturbance of the intracellular chloride regulation (Lux et al., 1970; Loracher et al., 1974), and changes in the transmembrane K^+ concentration gradient (Rausche et al., 1989) are all conditions that promote the generation of seizures. Glial cells may contribute to this disturbance by augmented uptake or consumption of GABA. Experimentally, GABA antagonists, drugs that block GABA synthesis and drugs that interfere with the GABA-dependent chloride currents by depression of Cl^- extrusion from cells, are capable of inducing seizures (Lux et al., 1978; Heinemann et al., 1989).

Similarly, the main excitatory neurotransmitter glutamate may be released in excess. This would occur when action potential generation occurs at branching points and in or near presynaptic endings at ectopic sites (Gutnick et al., 1972). Moreover, the number of excitatory nerve cell terminals may be increased (Sutula et al., 1988), the number, affinity, or gating mechanisms of glutamate receptors may cause increased postsynaptic currents (Walther et al., 1986), and changes in the transfer from dendritic sites of information pick up to the nerve cell soma due to changes in passive and/or active dendritic properties would also augment synaptic excitatory coupling (Kuno et al., 1970). Obviously, reduced glutamate uptake will increase extracellular glutamate levels and thereby eventually affect synaptic excitatory transmission. Synaptic stripping by microglia, the generation of dendritic action potentials and changes in the extracellular resistance, and thereby in the dendritic transfer function, will all cause augmented excitatory synaptic coupling and contribute to epileptogenesis. Indeed the excitatory action of glutamate has been discovered by showing that glutamate and its agonists precipitate seizures (Curtis et al., 1970). Activation of NMDA receptors

by reduced extracellular Mg^{2+} also contributes to seizure generation (Walther et al., 1986).

The balance of synaptic excitation and inhibition has a counterpart on the intrinsic cell level. Excitatory currents such as Na^+, Ca^{2+}, and unspecific cation currents are controlled by repolarizing voltage and Ca^{2+}-dependent K^+ or Cl^- currents. Again, blockade of these inhibitory currents by 4AP and TEA, for example, will precipitate epileptiform activity (Baranyi et al., 1979; Schwartzkroin et al., 1980) as do agents that remove inactivation of Na^+ currents. Chloride currents depend strongly on the transmembrane Cl^- concentration gradient. Agents that block Cl^- extrusion from cells cause an increase in intracellular Cl^- concentration and weaken Cl^- influx when Cl^- currents are activated, augmenting the risk of seizures. Indeed hydrazines and ammonium are well known to block chloride extrusion and contribute to seizure generation (Lux et al., 1986). A change in the transmembrane K^+ concentration gradient by reducing K^+ outward currents contributes to seizure generation and can, in some cases, underlie ictogenesis (Konnerth et al., 1984; Traynelis et al., 1988). Also, blockade of Ca^{2+}-dependent K^+ currents can be involved in the generation of seizures (Hablitz, 1981).

Finally, changes in the expression of ionic channels may be important to increase epileptogenicity. Thus increased expression of currents involved in burst discharges, such as Ca^{2+} and persistent Na^+ currents, may increase the number of burster cells in the central nervous system. Such changes, as well as downregulation in K^+ currents, will also augment synaptic input to dendrites and affect the dendritic integration properties.

Changes in ion homeostasis due to neuronal activity and alterations in oligodendrocyte or astrocyte function will eventually also contribute to augmented epileptogenesis. Thus reductions in $[Ca^{2+}]_0$ and $[Mg^{2+}]_0$ augment glutamate currents, particularly those mediated by NMDA receptors (Köhr et al., 1988) and increase neuronal excitability (Mody et al., 1988). Increases in $[K^+]_0$ will shift the reversal potential of glutamate currents to more depolarized levels, with a subsequent increase in glutamate current amplitude (Heinemann et al., 1990).

Glial cells can affect the balance between excitation and inhibition in many ways. The synaptic content of GABA and glutamate depends on glial metabolic activity. Thus glial cells may be stimulated to create more energy-rich particles. This will eventually lead to a reduction in the glial—and subsequently also neuronal—GABA pool. On the other hand, augmented release of glutamine will help nerve cells in the production of glutamate and thus contribute to seizure generation.

Glial cells may play a role in ictogenesis not only by affecting the extracellular glutamate and GABA levels and the ionic homeostasis. Glial cell swelling will decrease the extracellular space size and eventually augment extracellular ionic changes and transmitter levels. Decreases in extracellular space size have recently been shown to contribute to seizure recruitment (Dudek et al., 1990).

Glial cells, moreover, play an important role in detoxification. It is not inconceivable that during seizures free radicals are produced. The detoxification of free radicals depends on intracellular glutathione levels (Murphy et al., 1989; Vornov et al., 1991). Alterations in glutathione levels present in glial and nerve cells will affect the detoxification. This in turn may have effects on neuronal excitability. Production of nitric oxide and carbon monoxide is also dependent in some brain regions on glial function (Boje et al., 1992; Lafon-Cazal et al., 1993; Liu et al., 1993; Ruppersberg et al., 1993; Sagara et al., 1993). Since these agents are thought to promote presynaptic glutamate release (Stevens, 1993) this would affect neuronal transmission. They often also activate enzyme systems, which in turn affect neuronal excitability.

Between seizures the central nervous system often works completely normal, indicating that many of the above changes are secondary to conditions that result from a more or less arbitrarily occurring disregulation. One factor possibly of importance is the release of stress-related hormones such as corticotropin-releasing hormone or gluco- and mineralocorticoids (Nomizu et al., 1989; Joels et al., 1990; Baram et al., 1991). Effects of sexual steroids on GABA receptors (Harrison et al., 1987; Turner et al., 1989; Chvátal et al., 1991) may also play a role in such disregulatory behavior. Sleep-related changes in the release of catecholamines may be of similar importance (Chauvel et al., 1986; Wada et al., 1993).

PRINCIPLES OF SEIZURE GENERATION

Seizures are generally classified with respect to generalization (primarily, secondarily), behavior characteristics (absence, petit mal, grand mal), type of motoric symptoms (drop attacks, Jacksonian march, etc) and with respect to site of origin (temporal, frontal, simple, complex, etc). An important classification may also be based on pharmacological responsiveness. Thus, petit mal seizures are sensitive to drugs that block T-type Ca^{2+} channels (Coulter et al., 1989), while grand mal seizures are sensitive to

drugs that augment GABAergic function or reduce repetitive firing by interference with sodium channels (Macdonald et al., 1986). It seems that most anticonvulsant and antiabsence drugs have more than one action, a reason why these agents in fact can be used in a broad group of disorders.

Seizures can be induced by all drugs that interfere with the balance between inhibitory and excitatory mechanisms. The symptoms of such drug-induced seizures depend on the route of application. Systematically applied convulsants lead to primarily generalized nonconvulsant or convulsant activity. Focal application causes local disturbances with locally induced hyperactivity. The symptoms evolving from a seizure relate not only to consciousness and motor symptoms but also may affect emotional and memory functions. They depend on the area of disturbance. Thus analysis of seizures and of their morphological, electrophysiological and psychological correlates promote an insight into brain function.

When convulsants are systemically applied, the locus minoris resistentiae for induction of seizures seems to be the thalamus. When the dose is low, absence-like seizures ensue. These exploit local rhythm generating mechanisms of the thalamus. Thalamocortical projection neurons express transient Ca^{2+} currents. These are activated by depolarization. These channels show a voltage-dependent inactivation and a sufficient hyperpolarization both with respect to amplitude and duration is required for removal of inactivation (Carbone et al., 1984). Depolarization of thalamic neurons to about −50 mV sets the condition for rhythm generation. Then an inhibitory potential triggers a low threshold Ca^{2+} spike due to removal of Ca^{2+} current activation. This in turn elicits a burst of 1 to 3 action potentials (Jahnsen et al., 1984), which reach the cortex and the nucleus reticularis thalami (NRT). Each cell in the NRT generates long-lasting inhibitory postsynaptic potentials (IPSPs) in a large group of thalamic projection neurons. These IPSPs remove again the inactivation of Ca^{2+} channels and upon termination of an IPSP a new low threshold Ca^{2+} spike is generated with subsequent generation of sodium-dependent action potentials. The projected activity into the cortex causes activation of cortical columns. Their lateral connections and the projections back into the thalamus help in synchronization. Under these conditions cortical neurons display large IPSPs (Kostopoulos et al., 1983). This is why some authors consider these seizures as inhibitory in nature.

If the thalamus is activated more strongly, primarily generalized convulsant seizures ensue. These are no longer only characterized by augmented inhibition and by an abnormal synchronization but by high-frequency discharges (Gutnick et al., 1975; Heinemann et al., 1979). It was assumed initially that this activity represents the recruitment of the thalamus from the neocortex into seizure activity. However, it was later shown that seizure-like events can also be induced in isolated thalamic nuclei (Heinemann et al., 1979). Interestingly, stimulation of afferent fibers in the lemniscus does not cause seizure-like events in the thalamus. This may be related to the fact that such stimulation does not cause major ionic changes in the thalamus. Thus neither slow DC field potentials nor larger ionic changes of $[K^+]_0$ can be caused by afferent stimulation (Gutnick et al., 1979). The reasons for this resistance are not quite clear. It may be speculated that the particular organization of glial cells ensheathing synaptic glomeruli plays an important part in this protective role. Differences in expression of K^+ channels and the fact that all afferent fibers to the thalamus are relatively fast-conducting and well-myelinated may be important too.

Focal convulsant application in the neocortex leads to local interictal and ictaform events with typical associated DC and ionic changes. The interictal discharges depend intracellularly on unitary depolarizations of the neuronal membrane paroxysmal depolarization shift [PDS] (Matsumoto et al., 1964). These events are usually initiated in layer 4 to 5 where pacemaker cells have been found (Gutnick et al., 1982). They recruit the deep and if inhibition is impaired also related superficial neurons into the abnormal activity. The PDS represents therefore usually giant EPSPs with components of non-NMDA and NMDA receptor-mediated components.

The generation of seizures depends on the rapid succession of interictal discharges sometimes driven by peripheral input. Positive feedback can also be provided by backfiring of secondarily recruited areas in the zone of interictal activity (Jones et al., 1990).

Seizurelike events are characterized by prolonged depolarizations of neuronal elements to about −30 mV and sustained slow shifts of the extracellular field potential and the EEG. These slow shifts are superimposed by high-frequency low-amplitude transients during the tonic phase and large-amplitude low-frequency during the clonic phase. Intracellularly the clonic afterdischarges are similar to PDS superimposed on a slowly repolarizing membrane potential transient. Tonic and clonic activity refer to the fact that in a freely behaving animal or in man such episodes are associated with tonic and clonic muscle contractions, provided such activity has recruited the motor cortex.

The tonic phase represents an episode of sustained neuronal depolarization, while PDS-like afterdis-

charges underlie the generation of clonic-like electrographic events. Both seizure-like events and interictal-like activity can be studied also in *in vitro* slice preparations. Initially, such preparations displayed only interictal-like discharges. Meanwhile, a number of *in vitro* seizure models exist with a comparable seizure phenomenology as that observed in man and in experimental animal models.

IONIC CHANGES DURING SEIZURES

Primarily generalized and focal convulsions are characterized by marked changes in the ionic environment. During interictal discharges in intact animals rises in $[K^+]_0$ by up to 2 mM have been observed. $[Ca^{2+}]_0$ may decrease by 0.1 to 0.3 mM. However, not all interictal discharges are that large. Thus we find in entorhinal cortex slices that only the interictal discharges immediately before a seizure are associated with such ionic alterations. The interictal discharges before are associated with much smaller ionic changes. Likewise in *in vitro* models of hippocampal epilepsy only small rises in $[K^+]_0$ by about 0.3 mM are seen during recurrent short discharges considered by many as models of interictal activity. This might suggest that there are two types of interictal discharges: one which is a precursor of convulsant activity and one which represents a state of activity preventing development of ictaform activity. Indeed, blocking of generation of short recurrent discharges in the hippocampus sometimes leads to generation of seizurelike events (Swartzwelder et al., 1987).

These seizure-like events are accompanied by rises in $[K^+]_0$ to about 10 mM in the neocortex and to about 12 mM in the hippocampus. $[Na^+]_0$ may decrease by about 15 mM, $[Ca^{2+}]_0$ by up to 0.6 mM in iatrogenically induced seizures and by more than 1 mM during photically induced seizures in the photosensitive baboon. $[Cl^-]_0$ may decrease by up to 10 mM or increase during a seizure. Seizures are further associated with decreases in $[Mg^{2+}]_0$ and by acidic pH shifts. Each seizure is associated with a decrease in ES size by up to 50 percent in cats and by about 20 percent in rats. These decreases in ES size are due to transmembrane ion fluxes. The difference in ES size changes might be related to differences in the average length constant of a glial syncytium. The glial syncytium in cat neocortex is assumed to have a longer length constant than that in rat neocortex (Gardner-Medwin, 1981).

The ionic changes can be shown to produce a number of effects that promote the generation of seizures. Rises in $[K^+]_0$ reduce often voltage, Ca^{2+}, and transmitter-activated outward currents including those activated by GABA via $GABA_B$ receptors (Rausche et al., 1989; Ficker et al., 1992). Rises in $[K^+]_0$ augment spontaneous transmitter release. Glutamate currents become more effective upon rises in $[K^+]_0$ due to a depolarizing shift of the reversal potential. Moderate increases in $[K^+]_0$ therefore promote excitatory synaptic transmission (Rausche et al., 1990). Decreases in extracellular Ca^{2+} augment neuronal excitability due to shifts in the steady-state activation behavior and reduced surface charge screening. Evoked synaptic transmission is also sensitive to reductions in $[Ca^{2+}]_0$. Synaptic inhibition tends to fail before excitatory synaptic transmission (Jones et al., 1987). In addition lowering of $[Ca^{2+}]_0$ has promoting effects on activation of NMDA receptors, a subclass of glutamate receptors, which may be important in some forms of epilepsy (Köhr et al., 1988). Decreases in extracellular space size are also important. Thus, it has been shown that lowering of $[Ca^{2+}]_0$ elicits seizure-like events in the CA1 field of the hippocampus (Haas et al., 1984). When the extracellular space is shrunken by appropriate measures, also other structures are capable to produce seizure-like events upon lowering of $[Ca^{2+}]_0$ (Dudek et al., 1990). Interestingly, seizure-like events produced by lowering extracellular Ca^{2+} persist when evoked synaptic transmission is blocked. Under this condition, seizure-like events can even spread. The mechanisms of seizure spread seem to depend on extracellular K^+ accumulation. Indeed, simultaneous measurements of rises in $[K^+]_0$ and local seizure onset show that rises in $[K^+]_0$ precede local seizure onset. Three arguments point to a role of potassium in precipitation of low Ca^{2+} seizures: When $[Ca^{2+}]_0$ is lowered in the presence of low potassium levels the point of seizure onset occurs later or not at all. When seizures are induced by local stimulation, seizures commence only when $[K^+]_0$ is increased to between 5 and 6 mM. Finally, local injection of K^+ induce seizure-like events only when $[K^+]_0$ is increased to levels near 5 mM. These findings suggest that seizure spread under conditions of blocked chemical synaptic transmission occurs due to potassium release from activated cells into its surround. Indeed, the rises in $[K^+]_0$ commence often long before seizures ensue and before local neuronal activity increases.

CONTRIBUTION OF SPATIAL K^+ BUFFERING TO K^+ REGULATION DURING SEIZURES AND GENERATION OF SLOW NEGATIVE FIELD POTENTIALS

It was quite early recognized that glial cells may serve a function in $[K^+]_0$ homeostasis (Kuffler et al.,

1966). During a single action potential, :$[K^+]_0$ increases by 0.01 to 0.02 mM (Heinemann et al., 1975). If cells fire with high discharge frequencies this would amount to a dramatic increase in $[K^+]_0$. For example, nerve cells fire during a seizure with frequencies up to 200/s for periods as long as a minute. Provided there were no K^+ regulation, this would amount to a K^+ accumulation of some 50 mM. Rises in $[K^+]_0$ during a seizure are limited to about 12 mM in the hippocampus and to about 1 mM in neocortical structures. Three mechanisms can contribute to the limitation of rises in $[K^+]_0$: Na-K-ATPase activation, KCl cotransport into glial cells and spatial redistribution of K^+ through glial cells from sites of maximal neuronal activation into the less active surround (Figure 62-1). In chronic epileptic tissue the glial sodium potassium ATPase seems to be altered (Grisar, 1984). For spatial K^+ buffering through glial cells three requirements are necessary:

1. K^+ elevations should not be homogeneous throughout all cortical layers. This is the case during seizure activity (Dietzel et al., 1980).
2. Glia cells must respond with a near Nernstian depolarization to elevations in $[K^+]_0$ (Lothman et al., 1975).
3. Glial cells must be spatially extended and/or electrically coupled.

Under these circumstances glial cells will locally be depolarized by K^+ accumulation. This depolarization will spread along the surface of glial cells and propagate electrotonically into neighboring cells through gap junctions. As a result the depolarization of the glial cell will be smaller than expected from the K^+ equilibrium potential and a driving force for K^+ uptake into glial cells will develop. At remote sites the glial cells will be depolarized more than expected from local K^+ accumulation and therefore K^+ will be released. Due to the uptake of K^+ into glia negative field potentials at the site of maximal K^+ accumulation will develop, while at sites of maximal K^+ accumulation a positive slow field potential will appear. The extracellular potential gradient leads to a current flow from remote sites into the active focus. This will be carried predominantly by the majority ions Na^+ and Cl^- according to the respective concentration and ion mobility. This results in a charge transfer with Na^+ ions moving to the site of

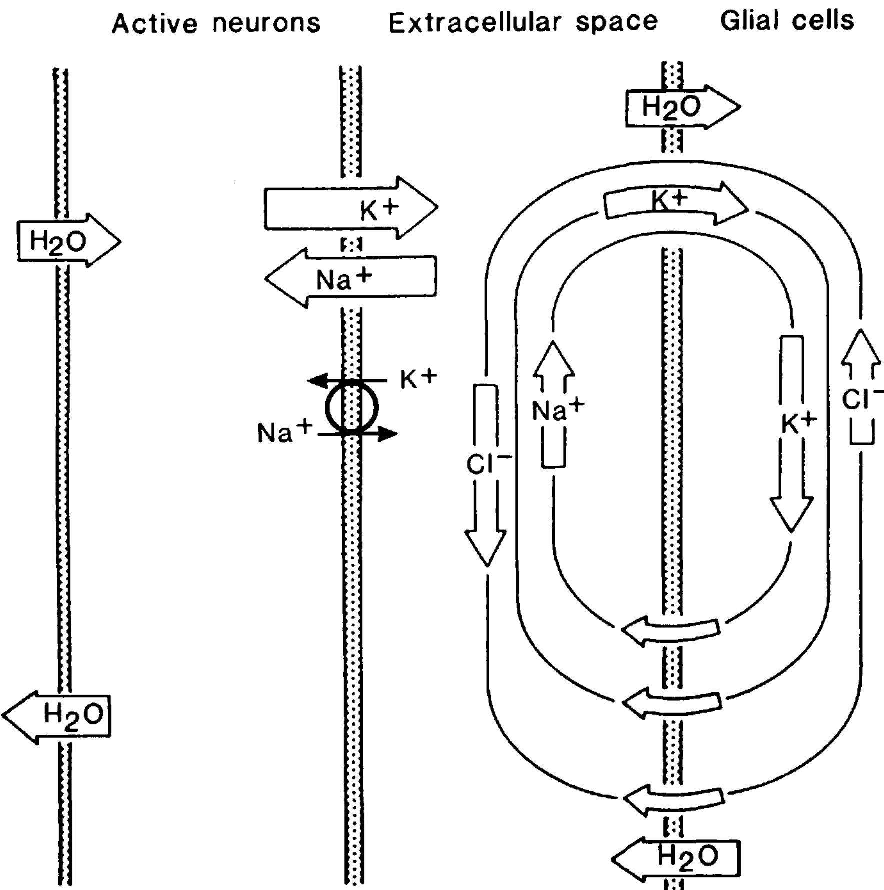

FIG. 62-1. Scheme of spatial K^+ buffering. *Open arrows* indicate concentration driven or current transport. The K^+ transport into glia creates a negative extracellular field potential, the K^+ release from glia creates a positive field potential. Due to current transport effects (current in biologic media is carried by cations and anions) the number of K ions taken up into glia is not fully substituted by Na ions. This results in osmotic imbalances leading to changes in the size of the extracellular space.

maximal K^+ accumulation and chloride ions moving away from such sites. As a result of this transport number effect not all K^+ ions moving into glia will be substituted by Na^+ ions and an osmolar imbalance develops, leading to water uptake into glia and shrinkage of the extracellular space (Figure 62-1).

A causal role for rises in $[K^+]_0$ in the generation of slow negative fp has been suggested by a number of observations:

1. Pharmacological treatments (barbiturates, phenytoin, etc), which reduced the changes in $[K^+]_0$, also reduced the amplitude of the slow negative fp's.
2. Experimental procedures which produced decreases in $[K^+]_0$ were associated with generation of positive fp (Heinemann et al., 1975).
3. Artificial increases of $[K^+]_0$ by iontophoresis, pressure application or superfusion always induced slow negative fp (Heinemann et al., 1984).
4. At sites where the negative fp were the largest, the changes in $[K^+]_0$ were also maximal that is, in the dorsal horn of the spinal cord (ten Bruggencate et al., 1974), the middle cortical layers of neocortex the stratum pyramidale of hippocampus (Benninger et al., 1980), the stratum granulare of the dentate gyrus, or the stratum moleculare in the cerebellum (Nicholson et al., 1978). As expected from the spatial buffer hypothesis slow fp's recorded at sites remote from those where the maximal increases in $[K^+]_0$ occurred were usually positive in polarity.

The extent by which glial cells contribute to the generation of slow fp is difficult to estimate because for many neuronal types long dendritic trees may be oriented in parallel to the K^+ concentration gradient in the tissue. This applies, for example, to the hippocampus and the neocortex. Prolonged depolarization of pyramidal cell somata may lead to a charge transfer into the passive dendrites with a current sink at the soma level and a current source at remote sites. We have recently therefore studied the relationship between the CSD density distribution of slow field potentials and changes in $[K^+]_0$ in rat hippocampal slices. We found that alvear stimulation resulted in two current sinks: one at the site of maximal K^+ accumulation and one in the stratum radiatum. Blockade of synaptic transmission by lowering $[Ca^{2+}]_0$ or by application of a cocktail of glutamate antagonists reduced the current sink in the stratum radiatum but left the current sink in the stratum pyramidale largely unchanged, that is, the layer where rises in $[K^+]_0$ were maximal. Conversely, when we reduced glial cell K^+ conductances by application of Ba^{2+} (Grafe et al., 1987) the current sink in the stratum pyramidale disappeared, while the current sink in stratum radiatum was still present (Figure 62-2). As predicted from the spatial K^+ buffer hypothesis glial membranes were in the stratum pyramidale slightly less depolarized than expected from the Nernst equation for the simultaneously measured rises in $[K^+]_0$ in this layer, while at remote sites the transmembrane glial potential was bigger than expected for a Nernstian depolarization of glial cells with the local measured rises in $[K^+]_0$ (Albrecht, Luxemburger-Weber, Leweke, and Heinemann, in preparation).

K^+ REDISTRIBUTION THROUGH GLIAL CELLS DURING EPILEPTIFORM ACTIVITY

A further analysis of the current source distribution was therefore made with respect to the question how much potassium could be shifted through the glial syncytium during seizures in cat neocortex and rat

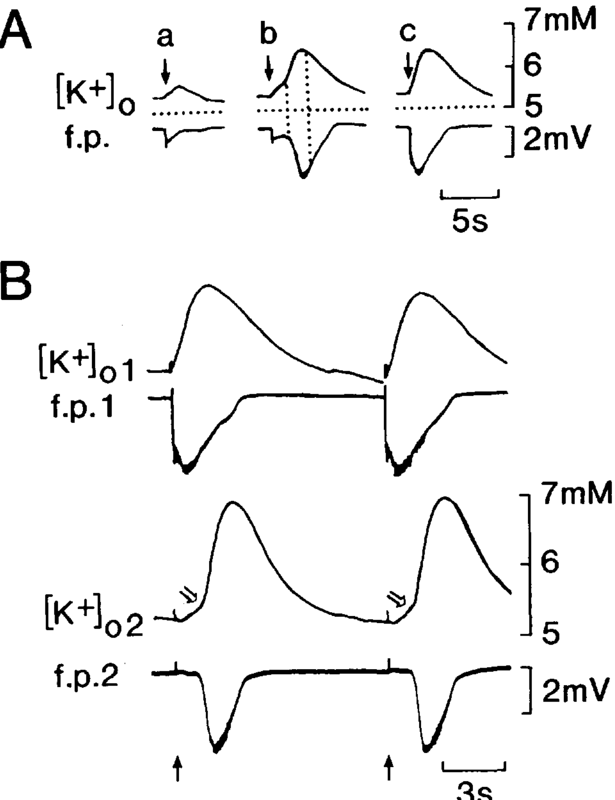

FIG. 62-2. Induction of low Ca^{2+} seizures depends critically on extracellular K^+. (A) Stimulus-induced discharges develop into spreading discharges only when critical K^+ concentration is surpassed. Recording in area CA1 with a K-sensitive microelectrode during nearby stimulation of SP. *a–c*: increasing stimulus intensities. (B) Simultaneous records of single stimulus-induced spreading epileptiform discharges in rat area CA1. Note that the discharge at recording position 2 commences considerably after the onset of the first discharge. The secondary response is preceded by a rise in extracellular $[K^+]_0$.

hippocampus. While in cat cortex this amounts to about a millimole per second, the value was considerably higher in the hippocampus.

To test the effectiveness of spatial K^+ buffering during low Ca^{2+}-induced epileptiform activity, we measured the changes in $[K^+]_0$ occurring during these seizures in the hippocampal area CA1 and the functionally uncoupled but neighboring dentate gyrus (Figure 62-3). As during stimulus-induced seizures, rises in $[K^+]_0$ were largest in the stratum pyramidale of area CA1 with a decline in amplitudes toward stratum radiatum. However, transient changes in $[K^+]_0$ could still be measured in the dentate gyrus (Albrecht et al., 1989). This suggests that spatial K^+ buffering is effective in redistributing $[K^+]_0$ in the three dimensions of space.

The interesting aspect of this type of activity is that it can still spread, although this type of activity persists under conditions where synaptic transmission is blocked. At sites that are secondarily recruited into seizures, the seizures are preceded by rises in $[K^+]_0$. It is possible that such rises are sufficient to trigger epileptiform activity. Indeed, artificial focal application of K^+ can trigger a spreading epileptiform event.

It seems that in addition to spatial buffering the generation of slow field potentials is also involved in the spread and induction of this type of activity. This is suggested by the finding that the field potential gradients are in the order of 40 mV/mm. Such gradients are experimentally sufficient to excite cells as has been shown for the dentate gyrus and the cerebellum (Jefferys, 1981; Hounsgaard et al., 1989). The field potential gradients can be reduced by superfusing the slices. This will short-circuit the field potential gradients along the dendritic trees of hippocampal pyramidal cells. It can be shown that such maneuvers block the low Ca^{2+}-induced epileptiform activity. Thus it appears that spatial K^+ buffering by redistribution of $[K^+]_0$ and by contribution to generation of slow field potentials contributes to the nonsynaptic spread of seizure activity. This contribution will, however, vary dependent on the spatial voltage gradients associated with K^+ buffering. These are in the order of 10 to 12 mV/mm in the thalamus and less than 10 mV/mm in the neocortex but may increase under conditions of brain edema.

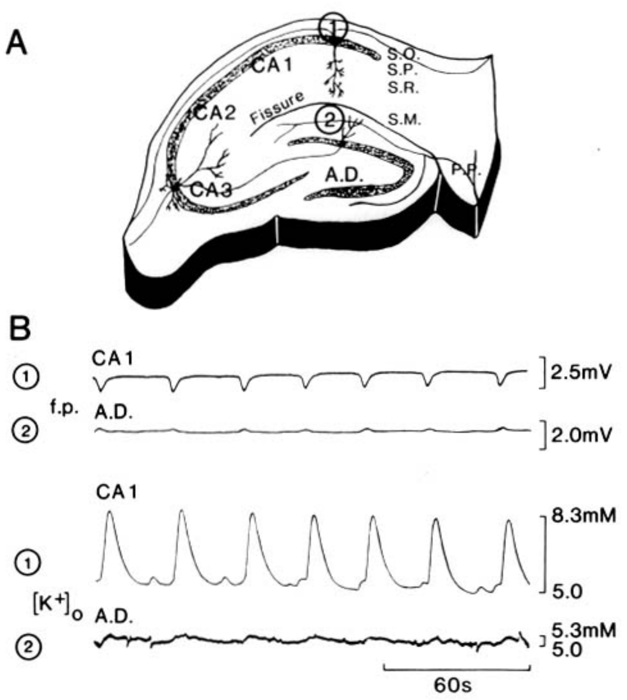

FIG. 62-3. Simultaneous record of seizure-like events in area CA1 and the dentate gyrus. The dentate gyrus does not actively participate in the SLEs. Yet each positive field potential in the dentate gyrus is associated with a small increase in $[K^+]_0$. Recording positions as in the inset.

SPATIAL K^+ BUFFERING IN CHRONIC EPILEPTIC TISSUE

That the gliosis often associated with an epileptogenic area contributes to seizure generation is suggested by the fact that surgical removal of such areas cures often the epileptic disorder. Indeed, the clinical observation of a shrunken hippocampus is taken as evidence for a seizure initiation site.

Since chronic epileptic foci are characterized by a considerable gliosis the question arises whether gliosis contributes to epileptogenesis by failure in K^+ regulation. This is very likely not the case. In glial tissue of this type K^+ accumulation is normal. The generation of slow field potential shifts by artificially increasing extracellular potassium still works. Also an indirect test for spatial K^+ buffering suggests that these cells are probably efficient in buffering extracellular K^+. This test is based on the transport number effect (Gardner-Medwin, 1980). If a DC current is applied through the cortex, ions move with the current according to mobility and concentration. Measurements of changes in $[K^+]_0$ show that the change in $[K^+]_0$ is much larger than expected from the transport number. This can be explained when it is assumed that some of the current moves through the relative low ohmic glial cells which express predominantly K^+ channels in their membrane. If such a test is applied to gliotic tissue the observed changes in $[K^+]_0$ are somewhat larger than in normal tissue suggesting that glial cells buffer potassium more ef-

fectively. This may very well facilitate the spread of epileptiform activity in such tissue.

The situation may, however, be different in chronic temporal lobe epilepsies with a sclerotic hippocampus. This is characterized by a profound gliosis. Recently, it has been reported in one model of chronic temporal lobe epilepsy produced by application of kainate that the reactive astrocytes are positive for markers of astrocytes type II (Represa et al., 1993). This marker indicates in tissue culture conditions and in the rat optic nerve astrocytes derived from a common precursor cell with oligodendrocytes. Whether the properties of such astrocytes differ with respect to K^+ regulation is unknown. One might also hypothesize that these cells have different properties for glutamate and GABA uptake and different capability for release of excitability modulating agents.

EPILEPTOGENESIS IN JUVENILE TISSUE

The readiness of a given area of the brain to generate seizure-like events varies with age. Thus it has been shown in *in vitro* preparations that seizure-like events are more readily generated in juvenile than in adult tissue. These seizure-like events are accompanied in the neocortex and hippocampus by abnormally large rises in $[K^+]_0$ (Hablitz et al., 1987; Hablitz et al., 1989). We have looked into the hippocampus to see whether this is related to any morphological alterations. It was found that GFAP-positive astrocytes are in the first week postnatally mostly restricted to the alveus and the molecular layer. The stratum radiatum has normal cells numbers for GFAP positive astrocytes at about 2 weeks after birth, while close to the stratum pyramidale it takes about 3 weeks to reach adult cell numbers (Nixdorf, Albrecht, and Heinemann, in preparation). However, in this age there is still abnormal K^+ accumulation, suggesting that glial cells are still unable to buffer extracellular potassium concentration. This might be related to a delayed maturation of glial cells in terms of expression of K^+ channels. Moreover, there is evidence that young astrocytes express glutamate and GABA receptors, with the possible result that the cells are depolarized by neurotransmitters (Blankenfeld et al., 1991; Steinhäuser et al., 1992). This would interfere with spatial K^+ buffering. When we tested for responses in gliotic tissue following exposure to kainate or in gliotic tissue near a scar focus we found however no effects of glutamate and GABA on extracellular ionic changes suggesting that spatial K^+ buffering is not hampered by such affects in gliotic tissue (Heinemann et al., 1984).

GLIAL CELLS AND pH REGULATION

Recently, there was considerable interest in the regulation of extracellular pH. It is now clear that glial cells contribute to changes in pH (see Chapter 14). Changes in pH in turn promote or reduce the probability of seizure generation. Thus alkalosis is proconvulsant by promoting activation of NMDA receptors and of voltage regulated ion channels. Acidosis in turn is considered to be anticonvulsant (Caspers et al., 1987). So far, nobody has looked at whether pH regulations is altered in chronic epileptic tissue. However, alterations in carbon anhydrase properties have been reported for gliotic tissue (Guillaume et al., 1991).

PROPERTIES OF GLIAL CELLS FROM DIFFERENT TYPES OF EPILEPTIC TISSUE

Surgery from epileptic patients has provided us with human material coming directly from such patients. It is possible even from poorly preserved specimens to cultivate glial cells. If intracellular Ca^{2+} is monitored in such cultures spontaneous and glutamate-induced oscillations are observed, which spread through the glial syncytium. The claim has been made that such Ca^{2+} waves differ in cultures coming from the surround of tumors and from epileptic tissue. However, convincing statistical material has not yet been presented.

STATUS EPILEPTICUS AND GLIAL CELLS

One of the biggest risks in terms of mortality for a patient who suffers from epilepsy is that he develops a convulsant status epilepticus. Usually a status epilepticus can be well controlled by immediate infusion of phenytoin or benzodiazepines. However, if the condition lasts for some time the risk is that the seizures become pharmacologically uncontrollable with presently available drugs. This condition can be mimicked in cortical slice preparations. If seizure-like events are induced by lowering extracellular Mg^{2+} and subsequent facilitated activation of NMDA receptors for glutamate seizure-like events develop in entorhinal cortex slices and in the neighboring subiculum as well as in the neocortical portions of the temporal lobe (Dreier et al., 1991) (Figure 62-4). These seizure-like events recur very frequently and thus mimic a status epilepticus. Initially these seizure-like events respond to classical anticonvulsants. However, after some time of exposure to low Mg^{2+}-containing artificial cerebrospinal fluid interictal discharges and clonic like after

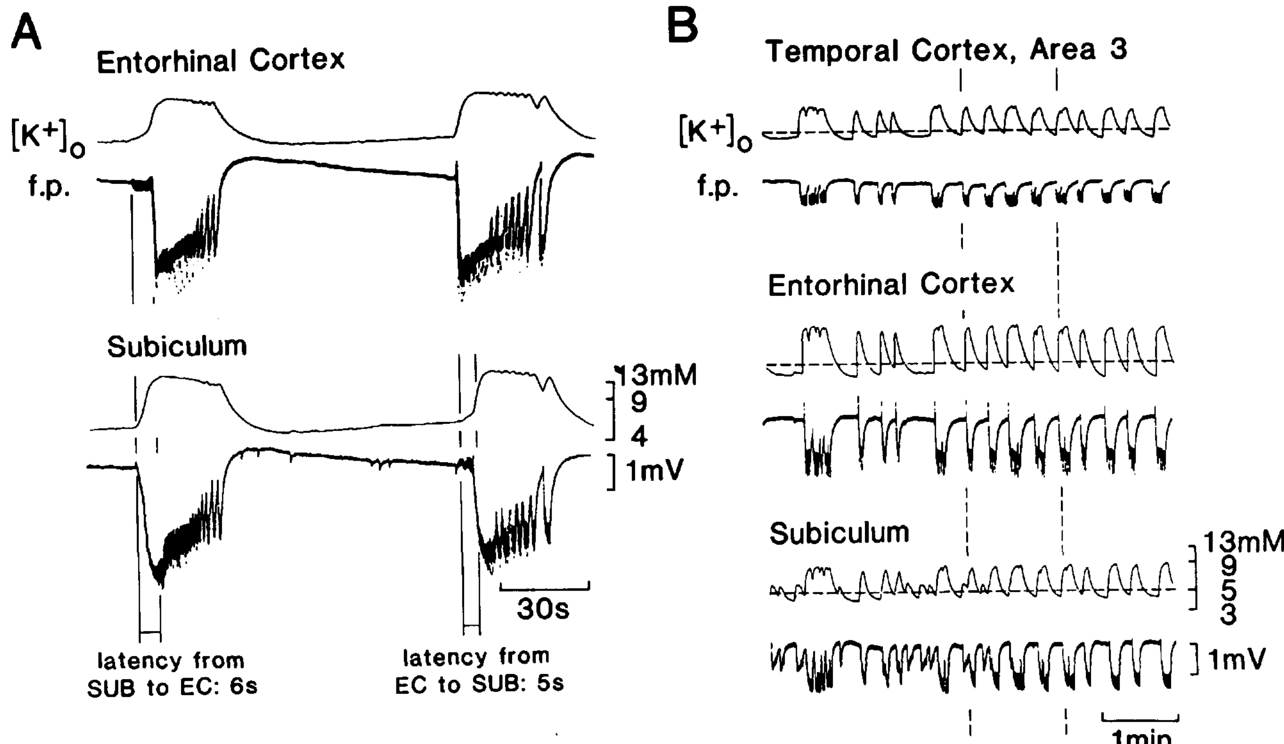

FIG. 62-4. Different patterns of epileptiform discharges in rat entorhinal cortex slices. SLEs were induced by lowering $[Mg^{2+}]_0$. (A) SLEs characterized by tonic- and cloniclike electrographic discharges. Note changing focus from the subiculum to the EC. Note that each structure, before being recruited into seizure activity, shows a rise in $[K^+]_0$. (B) Late epileptiform discharges in the entorhinal cortex slice with simultaneous activity in three different areas. This activity does not respond to clinically employed classical anticonvulsants, but responds to GABA and GABA uptake blockers.

discharges disappear. The frequency of discharges increases dramatically. These events are no longer controlled by presently marketed anticonvulsants (Heinemann et al., 1991). They also do not respond to AMPA-type receptor antagonists, although they are still sensitive to NMDA receptor antagonists. This condition can be induced immediately when GABA receptor antagonists are applied together with the low Mg^{2+} medium. This suggests that a failure in GABAergic neurotransmission is involved in the transition from the drug sensitive to the "drug-resistant" form of status epilepticus. Indeed, drugs that augment postsynaptic GABA actions, such as barbiturates and benzodiazepines, are ineffective in controlling these seizures. However, GABA, the $GABA_A$ receptor agonist muscimol and the $GABA_B$ receptor agonist baclofen can still block this activity. Thus changes in the efficacy of $GABA_A$ receptors to mediate inhibition are likely not responsible for the transition to drug-resistant status epilepticus-like activity. Since GABA uptake blockers are able to reverse and eventually block the difficult to treat status epilepticus it can be assumed that failure in GABA release is involved. This could be due to transfer of GABA from presynaptic endings into glia. Under the enormous metabolic demand during seizure activity some of this GABA may be burnt in the GABA shunt for production of ATP. It is likely that glial cells by removing synaptically released GABA play an important role in the transition from drug sensitive status epilepticus to a condition where presently marketed drugs fail to block this activity. The enormous cell swelling involving glial cells may in addition be a cofactor in the generation of this life-threatening condition.

CONCLUSIONS

Glial cells are abundant in the brain and often show distinct morphological alterations in an area of epileptogenesis. Thus brain injury often leads to gliosis in the surround of the scar. In this region nerve cells undergo regenerative and degenerative alterations, which are essential in generation of abnormal excitability. Similarly, supposed areas of epileptogenesis in the temporal lobe are characterised by reactive gliosis. These alterations of glial cell morphology include changes in GFAP positive processes and possibly also increases in numbers. Based on the assumption that glial cells are involved in potassium homeostasis it

was suggested that glial cell function in such areas is impaired. This disturbance of glial cell function was thought to render them incapable of maintaining normal potassium concentrations in the central nervous system. This in turn should lead to abnormal potassium accumulation with the result that seizures are generated. However, present evidence suggests that this is not the case. However, spatial K^+ buffering in normally functioning cells may well contribute to spread of epileptiform activity.

Since the development of modern glial research many more functions of glial cells have been elucidated. Thus it was shown that glial cells may be involved in transporting nutritional and other metabolic elements in and out of the brain across the blood-brain barrier. They serve many metabolic functions, and supply a major part of brain energy. In doing that, they use the GABA shunt for energy production; this may, during abnormal hyperactivity, very well lead to burning of GABA and thereby reduction of available GABA. Since GABA is the most important central inhibitory neurotransmitter, this would lead to a reduction of GABA levels and thereby aggravate seizures.

Astrocytes are involved in uptake of neurotransmitters from the extracellular space. Thus reduced glutamate or increased GABA uptake may well be involved in seizure generation.

More recently it was discovered that astrocytes release cytokines. These are hormones which have activating functions not only on the immune system, including microglia but also have effects on nerve cells, which may promote generation of hyperactivity.

Glial cells also express growth factors and release them. Following seizures, this expression is increased. Whether such growth factors contribute to epileptogenesis is not quite clear. However, the possibility must be investigated.

Although many of the more recently established glial functions may be involved in epileptogenesis as well as in generation, spread and termination of seizures such involvement was in many cases not rigidly tested. In other cases the multitude of mechanisms of seizure generation and seizure spread have not been considered.

Although glial cells show marked alterations during epileptogenesis we know surprisingly little about their role in epileptogenesis, seizure initiation, seizure spread, and seizure termination. There is scattered information here and there but this information is still sparse. There are well-defined epilepsy models in which these questions can be studied.

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63 Hepatic encephalopathy

MICHAEL D. NORENBERG

Hepatic encephalopathy (HE) or hepatic coma represents the neurological consequence of liver failure or the shunting of blood away from the liver (portal-systemic shunt). HE is probably the best example of a clinical condition in which astrocytes play a key and perhaps dominant role in pathogenesis. It is also a disease that has provided important insights into ammonia and glutamate metabolism as well as contributions to the concept of glial-neuronal interactions. As will be discussed, ammonia is a prime candidate as the neurotoxin in HE, and the astrocyte is the cell where ammonia is primarily metabolized. Our working hypothesis has been that astrocytes are mainly affected by toxins in HE and that abnormal glial function disturbs the microenvironment leading to disordered neuronal activity.

CLINICAL FEATURES

Impaired neurological function is one of the most common events in terminal liver failure. HE is generally divided into acute and chronic forms. Acute HE (fulminant hepatic failure) results from diffuse massive liver necrosis, usually occurring in the absence of preexisting liver disease. It is seen following viral hepatitis, drug toxicity (acetaminophen, halothane, nonsteroidal antiinflammatory agents) and various hepatic poisons (mushroom/Amanita phalloides, volatile hydrocarbons). It presents with the rapid development of delirium, seizures, and coma, and has an extremely poor prognosis. The principal cause of death in fulminant hepatic failure is brain edema associated with increased intracranial pressure. The only currently effective treatment is liver transplantation.

Chronic HE, sometimes referred to as portal-systemic encephalopathy, is far more common, is seen with preexisting liver disease (usually cirrhosis), and is commonly precipitated by infection, gastrointestinal hemorrhage, excessive diuresis, electrolyte imbalance, high protein diet, constipation, and the use of sedatives and hypnotics. The clinical picture is characterized by altered mental state, change in personality, altered mood and behavior, diminished intellectual capacity, abnormal muscle tone with brisk deep-tendon reflexes, and often a Babinski response. A characteristic tremor (asterixis) is seen but is not diagnostic of HE. Seizures are relatively rare. Electroencephalographic (EEG) abnormalities are frequently found. The patients often display a disturbed diurnal rhythm with abnormal sleeping patterns. In late stages the patients hyperventilate, resulting in respiratory alkalosis. Ultimately, patients develop a disturbance in the level of consciousness ranging from confusion to stupor and coma. With adequate treatment, the patients usually recover, although irreversible syndromes are known (Norenberg, 1981).

PATHOLOGICAL CHANGES IN HUMANS AND EXPERIMENTAL ANIMALS

The main pathologic change in acute HE is brain edema. Grossly, the brain shows evidence of transtentorial and tonsillar herniation. Astroglial swelling dominates the microscopic picture in human (Kato et al., 1992) as well as in the majority of experimental animal studies (Norenberg, 1977; Traber et al., 1987; Swain et al., 1991) (Figure 63-1).

In chronic HE, edema is absent but astrocytic changes continue to be prominent. As originally reported by von Hosslin and Alzheimer (1912) and more definitively described by Adams and Foley (1953), gray matter astrocytes are the cells affected (protoplasmic astrocytosis, Alzheimer type II astrocytosis). The cells have enlarged, pale nuclei with peripheral margination of chromatin and often prominent nucleoli (Figure 63-2). In long-standing cases, intranuclear glycogen is found. The cells possess an increased amount of lipofuscin. Loss of glial fibrillary acidic protein (GFAP) is characteristic (Sobel et al., 1981). Whether there is a true astroglial proliferation is unclear (Norenberg, 1981). These glial changes correlate with blood ammonia level and clinical state (Adams and Foley, 1953; Norenberg, 1981; Martin et al., 1987). No significant or consistent neuronal morphological changes have

FIG. 63-1. Electron micrograph of a swollen astrocyte from an experimental model of acute HE. The cytoplasm is enlarged and electronlucent, with prominent membrane-bound vacuoles. Glycogen granules are scattered throughout the cytoplasm.

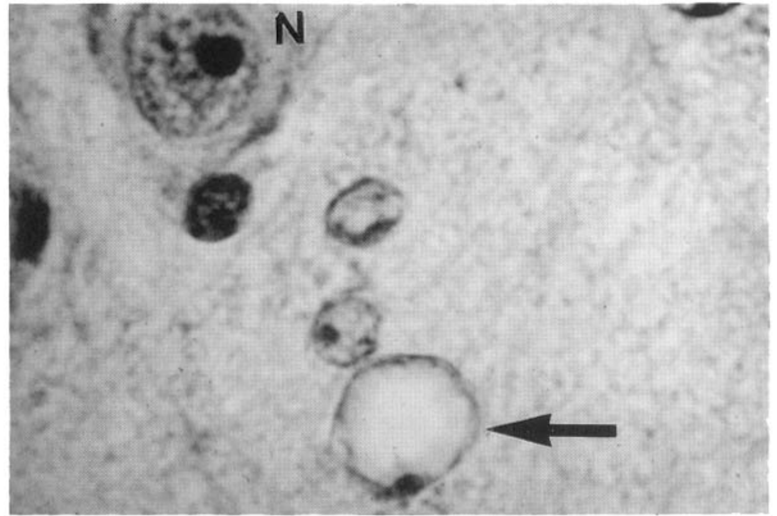

FIG. 63-2. Alzheimer type II astrocyte (*arrow*) showing nuclear enlargement and clearing, with the chromatin displaced to the periphery. Two adjacent relatively normal astrocytes are also present. A nearby neuron (*N*) is normal.

been identified (Adams and Foley, 1953; Norenberg, 1981; Martin et al., 1987).

Most experimental studies have shown great similarity regarding the astrocyte changes in HE. Initially, the cells show hypertrophy with increased number of mitochondria, smooth and rough endoplasmic reticulum, and an increase in cytoplasmic glycogen. As coma ensues, evidence of degeneration is observed characterized by increased cytoplasmic lucency (hydropic changes), the presence of cytoplasmic vacuoles and the occurrence of degenerated mitochondria. It was speculated that the early alterations were reactive ones, presumably due to hyperammonemia, whereas the later degenerative changes were a consequence of bioenergetic failure due to excessive glial involvement in ammonia detoxification. (For review see Norenberg, 1981).

PATHOGENETIC MECHANISMS

The pathogenetic mechanisms involved in HE are still unknown. Three views currently dominate: (1) the toxin/ammonia hypothesis and related disturbance in glutamate metabolism and neurotransmission, (2) abnormal GABAergic neurotransmission related to the presence of endogenous benzodiazepines or benzodiazepine-like compounds, and (3) the monoamine hypothesis. As developed in this article, these views are not necessarily mutually exclusive.

Ammonia

The role of toxins has dominated pathogenetic views for decades. This concept states that gut-derived nitrogenous products accumulate systemically because of failure by the diseased liver to clear these substances. A number of toxins have been incriminated; chief among these has been ammonia, whose actions have been extensively investigated. It is certainly the best known toxin, and remains to date the best candidate likely to play a key role in HE.

Evidence for the ammonia hypothesis is compelling. Arterial, cerebrospinal fluid, and brain levels usually correlate well with the clinical state (Conn and Lieberthal, 1978). Increased cerebrospinal fluid level of glutamine (a product of ammonia metabolism) is among the best indicator of encephalopathy (Hourani et al., 1971). Blood ammonia levels (usually venous) sometimes do not perfectly correlate with the clinical state. This lack of precise correlation has been the biggest drawback in unequivocally accepting the importance of ammonia. However, reasonable explanations have been provided that deal with these occasional discrepancies (Conn and Lieberthal, 1978).

Factors that lead to increased levels of blood or brain ammonia (administration of ammonium salts and resins, high protein diet, gastrointestinal bleeding, hypokalemia, constipation) all make HE worse. Indeed, procedures geared toward reducing blood ammonia levels form the basis for the treatment of HE. Further linking ammonia to HE are reports that patients with hereditary hyperammonemia have similar clinical and pathological findings as in HE. Lastly, experimental procedures that increase blood or brain ammonia, reproduce the same astrocytic changes present in HE.

Ammonia is derived mainly from the gut through the breakdown of nitrogenous substances by bacterial action. It is then carried to the liver where it is converted to urea. In disease states, where the liver is incapable of converting ammonia to urea, or in the presence of venous shunts resulting in portal blood bypassing the liver (portocaval shunts), ammonia reaches the brain where it is principally metabolized to glutamine, as the complete urea cycle is absent in brain (Sadasivudu and Hanumantharao, 1974). This action is carried out by glutamine synthetase, an ATP-consuming reaction, that is present mostly in astrocytes (Norenberg, 1983) (Figure 63-3). A reduction in brain glutamate and an increase in brain glutamine are important consequences of ammonia fixation. As discussed below, both of these effects of ammonia appear to play a major role in the pathogenesis of HE.

Glutamine released from astrocytes is taken up by nerve endings where it is converted to glutamate through the action of glutaminase (Bradford and Ward, 1976). Glutamate released from nerve endings is then picked up by astrocytes completing the so-called glutamate-glutamine cycle (Shank and Aprison, 1981). The key components of the cycle, namely, glutamate uptake, glutamine synthesis, and glutamine release have all been convincingly shown in astrocyte cultures (Waniewski and Martin, 1986; Farinelli and Nicklas, 1992).

Normally, glutamine synthetase works at maximal capacity (Cooper et al., 1985). Increases in GS activity are generally not found in hyperammonemia (Cooper et al., 1979). In fact, glutamine synthetase is decreased in portocaval-shunted rats (Butterworth et al., 1988a) and in human postmortem tissue (Lavoie et al., 1987a). Thus, the ability of the brain to fix ammonia in HE and other hyperammonemic states may be limited (Butterworth et al., 1988a). Work in cell culture fits with the above *in situ* observations in that no significant change is seen early on; however, with longer term treatment, glutamine

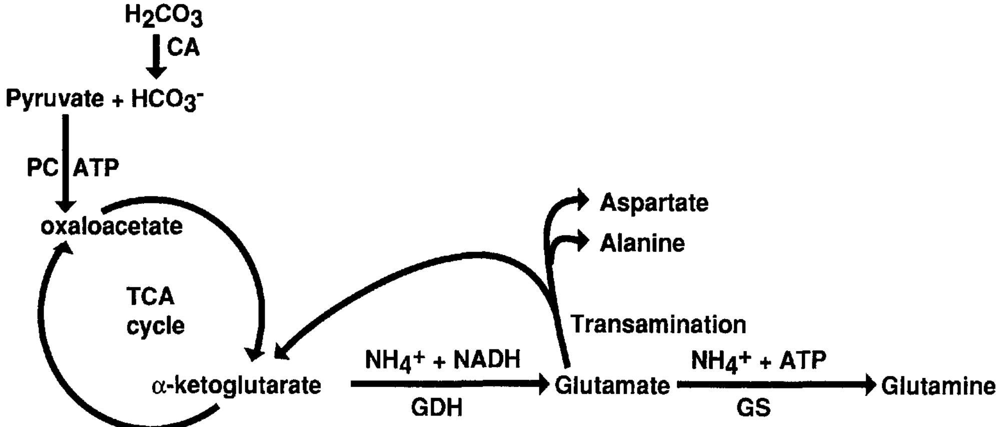

FIG. 63-3. Metabolic pathways of ammonia and glutamate metabolism. *CA*, carbonic anhydrase; *PC*, pyruvate carboxylase; *GDH*, glutamate dehydrogenase; *GS*, glutamine synthetase.

synthetase activity is significantly reduced (Norenberg et al., 1986). This may be indicative of an astroglial dysfunction, which may place the central nervous system at risk when challenged with prolonged high blood levels of ammonia.

Ammonia is clearly a neurotoxin (Cooper and Plum, 1987). High concentrations of ammonia result in seizures and death, while at lower concentrations, stupor, and coma predominate. The mechanisms of ammonia toxicity, however, are still not completely known.

The original view of energy failure proposed by the Bessmans (1955) remains highly controversial and, while having many detractors, cannot be totally dismissed (for review see Cooper and Plum, 1987). It would appear that acutely, there are no losses of high-energy metabolites (Hawkins et al., 1973); however, chronically, a component of energy failure is present. One possible mechanism is the inability of the malate-aspartate shuttle to generate reducing equivalents, secondary to an ammonia-induced fall in glutamate (Hindfelt et al., 1977).

Ammonia theoretically can alter the intracellular pH, an aspect that has been largely ignored in pathogenetic considerations. In most cells, ammonium chloride produces a transient rise in pH (Roos and Boron, 1981). The situation in brain is not clear. Portocaval-shunted rats show an increase in pH in astrocytes (Swain et al., 1991). However, nuclear magnetic resonance studies have generally shown no change in pH (Fitzpatrick et al., 1989) and one study even showed a fall in pH, presumably due to the generation of lactic acid (Brooks et al., 1989). Since pH can alter cell function (Häussinger, 1988), changes in intracellular pH may turn out to be one of the most important properties of ammonia.

Other effects of ammonia include inhibition of carbonic anhydrase and glutaminase, loss of vascular autoregulation, defective protein synthesis, enhanced carbon dioxide fixation, disinhibition of phosphofructokinase, fall in monoamine oxidase, and inhibition of Na^+,K^+-ATPase. Which of these changes may contribute to neurotoxicity and which are adaptive changes, is not clear. (For reviews and references see Norenberg, 1981; Cooper and Plum, 1987).

Ammonia may also interfere with glutamate-mediated excitatory neurotransmission. Brain glutamate levels in humans and in experimental animals are generally depressed (Hindfelt et al., 1977; Lavoie et al., 1987a; Dejong et al., 1992). Decrease in glutamate occurs in cultured astrocytes following ammonia treatment (Waniewski, 1992). The decrease in glutamate is largely due to glutamine formation during the process of ammonia detoxification.

Elevated levels of extracellular glutamate have also been described (Moroni et al., 1983; Therrien and Butterworth, 1991; Bosman et al., 1992). This elevation could be due to increased release of glutamate from neurons. At levels greater than 5 mM, ammonia stimulates glutamate release from synaptosomes (Erecinska et al., 1987). Other studies indicate that while basal levels of extracellular glutamate are not altered (Tossman et al., 1987), they increase following stimulation with high concentrations of K^+ (Tossman et al., 1987). Hippocampal slices from portocaval-shunted rats show increased glutamate release or "overflow" (Butterworth et al., 1991). The increase in extracellular glutamate could also be due to failure to take up glutamate. Ammonia inhibits synaptosomal glutamate uptake (Mena and Cotman, 1985) and work with astrocyte

cultures also indicate that ammoniated cells have a decreased capacity to take up glutamate from the medium (Norenberg et al., 1985). Sera and cerebrospinal fluid from patients with HE inhibit glutamate uptake in rat hippocampal slices (Schmidt et al., 1990). It appears that the increase in extracellular glutamate can come from increased neuronal release as well as decreased uptake.

Ammonia may also influence glutamatergic neurotransmission by downregulating glutamate receptors, possibly secondary to increased levels of extracellular glutamate (Peterson et al., 1990). Ammonia may also act directly on the postsynaptic aminohydroxymethylisoxazolepropionic acid (AMPA) receptor (Fan et al., 1990). However, the status of glutamate receptors remains unclear as increased binding to glutamate receptors in membrane preparations of galactosamine-treated animals were observed by Ferenci et al. (1984), while Zimmermann et al. (1989) did not observe such changes in rats treated with thioacetamide. Whether these discrepancies reflect species differences, model differences, or variations in the timing of the experiments, remains to be worked out.

Not as much work has been done with other proposed toxins, including short-chain fatty acids, mercaptans, phenol, endotoxin, and "middle molecules." Some of these may act synergistically with ammonia. (For review see Zieve, 1987).

Ammonia/Hepatic Encephalopathy and Brain Edema

The mechanisms involved in the production of HE-associated edema are controversial. Vasogenic, that is, the extracellular accumulation of fluid due to a disturbance of blood-brain barrier (BBB), and cytotoxic (intracellular accumulation of fluid) mechanisms have been proposed.

Support for the vasogenic origin of brain edema in HE comes from Livingstone et al. (1977), Horowitz et al. (1983), and Zaki et al. (1984). The models used, however, are associated with hypotension and hypoglycemia, both of which are known to induce vasogenic edema. The response to mannitol and ineffectiveness of glucocorticoids in the treatment of HE-associated edema do not support a vasogenic process. Lack of evidence of blood-brain barrier dysfunction in man and animal studies (Knudsen et al., 1988; Roy et al., 1988; Bassett et al., 1990) suggests that factors other than breakdown of the blood-brain barrier are involved in the production of edema.

Animal studies have emphasized astrocyte swelling as a major component of the edema associated with HE (Norenberg, 1977; Traber et al., 1987; Swain et al., 1991). The swelling correlates well with levels of ammonia (Swain et al., 1992). Benjamin et al. (1978) showed that ammonia causes swelling in tissue slices and Ganz et al. (1989) demonstrated that the swelling was mostly confined to astrocytes. Cell culture studies of ammonia-treated astrocytes also show cell swelling (Norenberg et al., 1991). Taken together, there is strong evidence to indicate that the main mechanism for brain edema in HE is cytotoxic, that the fluid is largely in astrocytes, and that ammonia is an important factor in the production of edema.

Gamma-Aminobutyric Acid/Benzodiazepines

A major working pathogenetic hypothesis in HE deals with the role of excessive GABAergic tone in HE. It was originally believed that there was increased blood GABA and increased GABA and benzodiazepines (BZD) receptors in brain (Schafer et al., 1983). Subsequent studies showed no changes in brain GABA levels in humans and experimental animals (Butterworth and Giguere, 1986; Butterworth et al., 1988a; Zimmermann et al., 1989). No changes in GABA receptors were found in experimental animals (Mans et al., 1992; Zimmerman et al., 1989). Furthermore, no changes in BZD receptors were found in thioacetamide-treated rats (Zimmermann et al., 1989), or in portacaval-shunted rats (Maddison et al., 1987). No change in GABA or BZD binding sites were noted in human postmortem tissue (Rossle et al., 1989).

Against these negative findings is the observation that flumazenil, a BZD receptor antagonist, is capable of reversing HE coma (Ferenci et al., 1989). Improvement with flumazenil has also been observed in thioacetamide-treated animals (Gammal et al., 1990). These findings prompted Mullen and colleagues (1988) to propose that elevated levels of BZD ligands might be responsible for HE. Indeed, compounds with agonist activity on the BZD receptor were found in humans and experimental animals with HE (Mullen et al., 1990). One of these ligands appears to be diazepam binding inhibitor (DBI), an endogenous polypeptide capable of displacing BZD binding from both central and peripheral BZD receptors (Costa and Guidotti, 1991), and which has negative allosteric effects on the binding of GABA to the $GABA_A$ receptor (Ferrarese et al., 1987). Increased levels of DBI were identified in cerebrospinal fluid of patients with HE (Rothstein et al., 1989). Basile found that brain extracts from experimental

animals and humans with fulminant hepatic failure inhibited binding with flumazenil and identified these compounds as 1,4-benzodiazepines (Basile et al., 1989; Basile et al., 1991).

However, there are problems with the BZD hypothesis. No response to flumazenil were observed in various experimental animals (Zieve et al., 1987; Gammal et al., 1990; Van Der Rijt et al., 1990) and the clinical response to flumazenil has not been impressive in larger populations of patients (Van Der Rijt et al., 1989; Pomier Layrargues et al., 1990). Recent reports, however, indicate that the use of partial inverse agonists of the BZD receptor (Ro 15-4513, Ro 15-3505) may be helpful (Bosman et al., 1991; Steindl et al., 1991). The issue of benzodiazepines is a hotly debated one, which nevertheless represents a major working hypothesis at the present time.

In linking the potential role of GABA and BZD to astrocytes, it may be recalled that astrocytes also possess BZD receptors, although they are of the peripheral-type which have been found in tissues outside the central nervous system. Within the central nervous system, most of the receptors are found on glial cells (McCarthy and Harden, 1981), and as in other tissues, appear to be present chiefly on mitochondria (Itzhak et al., 1993). The functions of this receptor are poorly understood.

An important aspect of the peripheral-type BZD receptor is its involvement in the intracellular transport and metabolism of cholesterol and the production of neurosteroids (Mukhin et al., 1989; Papadopoulos et al., 1991). DBI and DBI-derived peptides, by activating the peripheral receptor, can generate the production of neurosteroids. These steroids can positively modulate the $GABA_A$ receptor (Romeo et al., 1992). This process can be blocked by PK 11195, a specific blocker of the peripheral receptor. The production of neurosteroids in astrocytes through stimulation of the peripheral BZD receptor provides a potentially important link between astrocytes and the function of the neuronal $GABA_A$ receptor.

Upregulation of the peripheral-type BZD receptor have been observed in ammonia-treated astrocytes (Ducis et al., 1989), in portocaval-shunted rats (Giguere et al., 1992) and in human postmortem tissue from encephalopathic patients (Lavoie et al., 1990). As this receptor is largely mitochondrial, it may simply reflect the increased number of mitochondria found in astrocytes in experimental HE (Norenberg, 1977) and in ammonia-treated astrocyte cultures (Gregorios et al., 1985). Alternatively, through the generation of neurosteroids this receptor may contribute to alterations in GABAergic tone. See below for potential involvement of the peripheral receptor in cell swelling.

Monoamines

Studies into the pathogenesis of HE in the 1970s and early 1980s were dominated by the false neurotransmitter hypothesis (Fischer and Baldessarini, 1976). The concept proposed that gut-derived false neurotransmitters either replaced the "true" neurotransmitters or that these false neurotransmitters (octopamine, tyramine, phenylethylamine) resulted in a depletion of dopamine and norepinephrine and a rise in serotonin. This view was subsequently modified to include elevations in aromatic to branched-chain amino acid ratio in the blood of patients with HE (Fischer, 1984). This change in the ratio allows for greater amounts of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) to enter the brain (James et al., 1978). As these amino acids are precursors for neurotransmitters, increased blood levels of aromatic amino acids were believed to result in abnormal levels of neurotransmitters. This concept was subsequently linked to ammonia by noting that the transport of aromatic amino acids across the blood-brain barrier was stimulated by exchange with brain glutamine (derived from ammonia). Abnormalities in the blood-brain barrier have also been proposed as a mechanism for increased central nervous system levels of tryptophan and its metabolites (Jessy et al., 1990).

However, increasing brain levels of octopamine or reducing norepinephrine did not result in coma (Zieve and Olsen, 1977); in fact, norepinephrine levels were found to be increased in thioacetamide-treated rats (Yurdaydin et al., 1989). Postmortem material did not disclose changes in norepinephrine, dopamine, or octopamine (Cuilleret et al., 1980). There is currently little support for the false neurotransmitter hypothesis.

Abnormalities in serotonin have long been suspected to play a role in HE. Increased levels of tryptophan and/or 5-hydroxyindolacidic acid (5-HIAA) in cerebrospinal fluid in humans and animals correlate very well with the level of encephalopathy (Ono et al., 1978). Increased serotonin turnover has been described in portocaval-shunted rats (Bengtsson et al., 1991) and increased levels of the serotonin metabolite 5-HIAA as well as tryptophan have been found in hyperammonemic mice with congenital ornithine transcarbamylase deficiency (Batshaw et al., 1988) and in urease-treated rats (Bachmann and Colombo, 1983). There are intriguing associations between serotonin and HE, and it is likely that it is involved in the pathogenesis of HE. It should be noted that tryptophan is a precursor of quinolinic acid, which has been implicated in HE (see below).

STUDIES IN CELL CULTURE

Mossakowski and coworkers (1970) were the first to demonstrate that treatment of astrocytes with ammonia, copper, and sera from humans and experimental animals with HE produced Alzheimer type II-like changes in culture. The metabolic pathways of ammonia in cultured astrocytes and its effects on glutamate metabolism and on bioenergetics have been studied in detail (Yudkoff et al., 1984; Hertz et al., 1987; Fitzpatrick et al., 1988).

Our work dealing with the morphologic effects of ammonia and its synergism with other toxins, the protective effect of carnitine and branched-chain amino acids, the effect of ammonia on various glial specific enzymes and neurotransmitter uptake, the role of cyclic AMP, changes in protein phosphorylation, and effects on the peripheral-type benzodiazepine receptor have been summarized in other articles (Norenberg et al., 1986; Norenberg, 1989).

Calcium

In view of the potential involvement of the peripheral benzodiazepine receptor in HE (see above) and since the peripheral benzodiazepine receptor may influence astroglial calcium homeostasis (Bender and Hertz, 1985), we examined the effect of ammonia on calcium flux (Neary et al., 1990). Three-day treatment with ammonia resulted in a 35% decrease in influx, and a decrease in calcium accumulation without a change in efflux. Preliminary findings using a calcium imaging system suggests that the free cytosolic calcium is also reduced. As calcium is important in the regulation of many cellular processes, such reduction in calcium may have profound effects on cell function. Consistent with a role of calcium in the effects of ammonia on astrocytes, are studies showing that increasing cellular calcium levels by treatment with extracellular ATP (Neary et al., 1989) results in the reversal of many of the ammonia effects, including morphology, cell swelling, and GFAP content (Neary and Norenberg, 1992; Norenberg et al., 1992).

Cytoskeletal Proteins

Loss of GFAP is one of the characteristic features of HE in humans (Sobel et al., 1981). We have observed that a 4-day treatment with 10 mM ammonium chloride decreased GFAP content in cultured astrocytes by 35% (Norenberg et al., 1990). The effect was dose-dependent and was less prominent in cells that had been maintained in dibutyryl cyclic AMP. An even more impressive finding was the almost total absence of GFAP mRNA after a 1-day treatment with ammonia (Norenberg et al., 1992). This loss of GFAP mRNA appears to be due to mRNA instability (Neary et al., 1993). These changes in GFAP and GFAP mRNA could be prevented by extracellular ATP (Neary and Norenberg, 1992). No change in vimentin mRNA was observed while a slight fall in actin was noted. The significance of changes in cytoskeletal proteins in ammonia-treated astrocytes is not clear, although as discussed below, these proteins may play a role in ammonia-induced cell swelling.

Astrocyte Swelling

Treatment of astrocytes with ammonia results in cell swelling (Norenberg et al., 1991). Using primary astrocyte cultures, we have initiated a number of studies aimed at understanding the cellular mechanisms underlying this process.

Calcium plays a major role in volume regulation (McCarty and O'Neil, 1992) and the involvement of calcium-dependent protein kinases in regulating astrocyte cell volume has been recently emphasized (Bender et al., 1992a, 1992b; Bender et al., 1993). We have shown that ammonia results in a decrease in calcium intracellular content (Neary et al., 1990) and that the addition of extracellular ATP, known to increase intracellular calcium levels (Neary et al., 1989), caused a significant reduction in swelling in ammonia-treated astrocytes (Neary and Norenberg, 1992).

Cyclic AMP diminishes the cytopathic effect of ammonia (Gregorios et al., 1986). This may be relevant to the issue of cell swelling as treatment of cultures with dibutyryl cyclic AMP have a smaller water content and volume regulate more efficiently (Bender et al., 1992b). The effect of cyclic AMP on volume regulation may be related to greater calcium currents (Barres et al., 1989).

Whether the abnormalities in the cytoskeleton contribute to cell swelling is highly speculative. Nevertheless, there is evidence that the cytoskeleton plays a role in ion transport (Solomon, 1989) and volume regulation (Cornet et al., 1993). Cyclic AMP-treated cells are more resistant to swelling and, interestingly, they have a greater amount of GFAP (Chiu and Goldman, 1985).

The benzodiazepine system may also contribute to swelling. An interaction between peripheral benzodiazepine ligands and diuretics have been described (Lukeman and Fanestil, 1987). We have examined the effect of peripheral benzodiazepine on ammonia-

induced astrocyte swelling (Norenberg and Bender, 1993). Treatment with the agonist Ro 5-4864 exacerbated the swelling whereas use of a peripheral antagonist, PK 11195, diminished the extent of swelling. How these benzodiazepine-induced changes are brought about is not known. Perhaps the involvement of the peripheral receptor in calcium homeostasis may be a factor (Bender and Hertz, 1985).

We have investigated the possible role of *myo*-inositol in astrocyte swelling as this compound is capable of acting as an osmolyte and has been implicated in the maintenance of intracellular osmolarity (Sherman, 1989). We have found that 3-day treatment of astrocytes with ammonium chloride (5 mM) decreased *myo*-inositol uptake by 50%. These findings are in keeping with a 50% reduction in *myo*-inositol content in patients with HE as determined by proton magnetic resonance spectroscopy (Kreis et al., 1992). Such a reduction in *myo*-inositol uptake may reflect an adaptive response to ammonia-induced swelling.

Methionine Sulfoximine

The concept that glutamine may be exerting a deleterious effect in HE is an important one. Based on the views of the Bessmans (1955) that ammonia may be deleterious through the process of ATP-consuming glutamine synthesis, Warren and Schenker (1964) described a protective effect of methionine sulfoximine (MSO) (an inhibitor of glutamine synthetase) against ammonia-induced seizures, despite the fact that MSO is a potent convulsant in its own right, albeit with a rather prolonged latent period (Proler and Kellaway, 1962). Gutierrez and Norenberg (1975) showed that MSO produced the identical changes seen in HE, namely, the Alzheimer type II change. In one regard that was not surprising, as MSO results in a striking elevation of blood and brain ammonia levels (Warren and Schenker, 1964). However, the so-called protective effect of MSO remained puzzling in that an agent that protected against ammonia, itself increased ammonia levels and produced the histological changes associated with ammonia.

There is currently a resurgence of interest in MSO and the possible detrimental effects of excessive glutamine levels following the publications by Brusilow and colleagues (Brusilow and Traystman, 1986; Takahashi et al., 1991). Hawkins and Jessy (1991) and Hawkins et al. (1993) have shown that the effects of ammonia on glucose utilization and neutral amino acid transport (tryptophan and leucine) can be reversed by MSO. Swain et al. (1992) showed a correlation between edema and brain glutamine levels.

In recent studies, our laboratory has examined the effect of MSO on ammonia-induced toxicity. MSO diminished the extent of ammonia-induced cytopathic changes, cell swelling, glycogen loss, and *myo*-inositol transport (Norenberg et al., 1993). It thus appears that even in an *in vitro* system, MSO has a protective effect. Whether all of these effects can be explained by an inhibition of glutamine synthetase remains to be determined. MSO results in a massive release of glutamine from astrocytes (Albrecht and Norenberg, 1990), increased glutamate uptake in brain slices (Rothstein and Tabakoff, 1985), increased brain glycogen content (Folbergrova et al., 1969; Gutierrez and Norenberg, 1977), and increased GFAP content *in vivo* (Yamamoto et al., 1989). Much is still to be learned about the mechanism of action of MSO.

Glycogen

Abnormalities in glycogen metabolism have been described in HE (Norenberg, 1981); early on, there seems to be a decrease in glycogen, whereas later, there appears to be an increment (Zamora et al., 1973). Such an effect on glycogen, a substance that is largely found in astrocytes (Ibrahim, 1975), could have a profound effect on energy metabolism in states of substrate deficiency. We have found that glycogen content in astrocyte cultures is reduced by ammonia in a dose-dependent manner (Dombro et al., 1993). The reduction in glycogen could be significantly diminished by treatment with MSO (Norenberg et al., 1993). Importantly, the ability of astrocytes to hydrolyze glycogen by beta-adrenergic stimulation was diminished. These findings indicate that ammonia interferes with glycogen metabolism and additionally may disrupt important interactions between astrocytes and neurons.

PATHOGENETIC INTEGRATION

The studies described above indicate that, while initially the astrocyte attempts to respond to the metabolic challenge posed by ammonia, serious effects occur that may impair the cell's ability to further respond to the toxic insult. The astrocyte is actively involved in ammonia fixation; this may or may not be harmful. With high ammonia doses the astrocyte may not be able to adequately remove sufficient ammonia; the "spillover" may have direct neuronal effects or lead to deleterious effects to astrocytes. Glutamine synthetase activity is ultimately diminished, leading not only to further increases in tissue ammonia, but to the potential toxic consequences of

elevated glutamate levels. The latter may not only have effects on neurons, but is also capable of causing astroglial swelling (Kimelberg and Ransom, 1986).

Abnormalities in glutamate homeostasis and glutamate neurotransmission induced by ammonia represents a common theme in many views regarding HE. Whether it is due to failure of glutamate uptake, disordered glutamate metabolism, or abnormal glutamate receptor function remains to be established. With regard to glutamate receptors, the defect could possibly reside in glial cells, which have both ionotropic and metabotropic types of receptors. Clearly, there is still much to be learned regarding the role of impaired astrocytic ammonia and glutamate metabolism in the pathogenesis of HE.

If there is excessive glutamate release or content in the extracellular space, one needs to consider the possibility of excitotoxicity as a pathogenetic mechanism (Moroni et al., 1983). In keeping with this view, are the protective effects of MK-801 and AP-5 (Marcaida et al., 1992), NMDA receptor antagonists, on acute ammonia toxicity.

The potential involvement of quinolinic acid in HE is pertinent. Quinolinic acid, a tryptophan metabolite, is an agonist of the NMDA receptor (Stone and Perkins, 1981) and increased brain levels were reported in portocaval-shunted rats (Moroni et al., 1986a) and in the cerebrospinal fluid of humans with liver failure (Moroni et al., 1986b). It should be noted that the enzymes involved in quinolinate metabolism appear to be largely located in astrocytes (Kohler et al., 1987; Okuno et al., 1987).

Glycine has allosteric effects on the NMDA receptor (Fagg and Baud, 1988). Cerebrospinal fluid and brain glycine increase following hepatectomy (Tossman et al., 1987; Bosman et al., 1992). Kohno et al. (1990) reported hyperglycinemia in cirrhosis, which correlated with HE. Yudkoff et al. (1986) have described a robust glycine uptake by astrocytes. Possible failure of astrocytes to take up glycine, could result in excitotoxic injury.

The GABA/benzodiazepine hypothesis also involves astrocytes. Ammonia-treated astrocytes have a diminished capacity for GABA uptake (Norenberg et al., 1986). The upregulation of the peripheral-type benzodiazepine receptor was commented upon above. Of considerable interest is the production of neurosteroids that may impact on the neuronal GABA receptor (Papadopoulos et al., 1992; Romeo et al., 1992).

While the present focus on HE has been centered on ammonia/glutamate and benzodiazepines, abnormalities in monoamines are still a factor to contend with. Here, too, astrocytes may be involved. Receptors for norepinephrine and serotonin exist on astrocytes (Murphy and Pearce, 1987). Adrenergic receptors may be involved in HE as it has been reported that HE can be precipitated with propranolol (Nieminen and et al., 1988), a beta-adrenergic blocker, although this issue is controversial (Cales et al., 1989). Monoamines can be actively taken up by astrocytes (Katz and Kimelberg, 1985). Contributing to possible derangements in this system is the reduction in isoproterenol-stimulated cyclic AMP levels (Liskowsky et al., 1986), abnormal protein phosphorylation (Neary et al., 1987), and inhibition of monoamine oxidase activity (Norenberg et al., 1988).

Related to the issue of monoamines is a disturbance in the function of the blood-brain barrier, leading to abnormal levels of amino acid precursors of neurotransmitters. As astrocytes appear to be involved in the regulation of endothelial function (Janzer and Raff, 1987), a disturbance in astrocytes could contribute here as well.

Figure 63-4 illustrates the relationship of various pathogenetic mechanisms in HE to astrocytes.

CONCLUSIONS

This review has focused on the role of astrocytes in the pathogenesis of HE. This is not to say that cells other than astrocytes are not involved. Ultimately there must be neuronal effects for clinical disease to express itself. However, the role of these other central nervous system components is less clear.

While the precise mechanism of HE is still not known and the existing views are controversial, it is clear that the astrocytes play a key role in this clinical disorder. We have attempted to advance the concept that hepatic encephalopathy represents a primary gliopathy, perhaps leading to an interference with glial-neuronal communication. Such an astro-

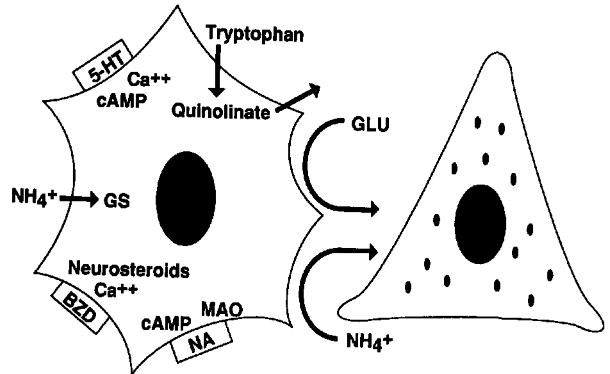

FIG. 63-4. Schematic diagram of various potential mechanisms involved in hyperammonemia and hepatic encephalopathy.

cyte injury could result in impaired neuronal and perhaps endothelial function.

Astrocyte swelling remains a major clinical problem whose pathogenesis is still poorly understood. Important new insights have evolved in recent years that promises to provide new leads into its cause and treatment. A better understanding of the role of calcium, cytoskeletal proteins, benzodiazepine receptors, and organic osmolytes should help in this regard.

The study of hepatic encephalopathy has provided a means for better understanding of the role of astrocytes in central nervous system function. This approach should continue to yield further insights into critical issues such as their involvement in neurotransmitter metabolism, detoxification, glial-neuronal interactions and other neurological disorders.

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64 Glial response to brain ischemia

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Glial cells, once considered to be mere supportive cells that nurtured the more robust activities of adjacent neurons, are now recognized as potentially the most dynamic cells within brain. Each glial phenotype appears to possess its own unique medley of channels, transporters, receptors, and enzymes (Murphy, 1993). Each can release and respond to a variety of organic and inorganic ions, lipids, and peptides (Kimelberg, 1988). Even cells of the same phenotype may be functionally different because of unique environmental conditions. Little is known about how these glial phenotypes interact during normal or pathologic conditions.

One actively studied area of glial function is their response to brain injury, including that following ischemia. After an ischemic insult, astrocytes and microglia undergo characteristic morphological and physiological changes and upregulate many proteins (for review see Eddleston and Mucke, 1993). Although the mechanisms underlying these changes remain poorly defined, alterations are likely in early response genes, cytokines, cytoskeletal elements, adhesion molecules, and enzymes. Frequently, changes in these parameters can only be surmised, based on their known roles in other cells outside of the central nervous system.

This chapter is not an exhaustive review of all up- and downregulated glial molecules following ischemia, important as they may be. Instead, we attempt to relate many important physiological observations made primarily *in vivo*, but also *in vitro*, to current theories of ischemic brain injury. We will take a decidedly systems approach. No brain cell exists in isolation. Glial cells potentially interact with each other, with the vasculature, with bloodborne elements, and with products from still other organ systems. If maintenance of normal function requires interaction between cells and their environment, it follows that degeneration, and possibly the lack of regeneration from ischemic injury, also depend on such interactions.

ASTROCYTES MAY AGGRAVATE ISCHEMIC BRAIN INJURY

How astrocytes influence the development of the brain's response to ischemic injury may be one important example of interactive cellular behavior in brain. Depending on the nature of the ischemic insult, astrocytes may in one instance be responsible for the destruction of brain tissue associated with infarction, and in another may serve to protect neutral cells from ischemic challenge and milder perturbations, such as spreading depression. The spectrum of astrocytic responses might be directly related to their role in ion homeostasis, and in addition seems to depend on their unique energy capacities. Their handling of two ions in particular, H^+ and Ca^{2+}, seems most relevant to the development of the tissue's response to ischemic insult.

A global reduction in blood flow of minutes duration destroys selectively vulnerable neurons (for review see Graham, 1992). Yet when brain glucose content is elevated shortly before the onset of global ischemia, all brain cells can be destroyed with the development of pan necrosis (i.e., infarction) (for review see Plum, 1983). Since glucose is converted to lactic acid under such conditions (Krebs, 1975), many have assumed that excessive acidosis is a necessary concomitant for the development of brain infarction. Glycogen is preferentially found in astrocytes within adult brain. If glycogen is a source of glucose for astrocytes, Plum (1983) reasoned that astrocytes might accumulate both hydrogen ions and osmols during ischemia. The latter conceivably could result in astrocytic acidosis and swelling. Supporting evidence for this supposition came from histological studies that show marked swelling and ultimate disruption of astrocytic cell membranes within zones of infarction from hyperglycemic, global ischemia (for review see Plum, 1983). The mechanisms by which astrocytes swell from ischemia remain largely inferential (Kimelberg et al., 1992). Furthermore, the carbohydrate source for increased lactic acidosis is also unknown. However, evidence is available to suggest that astrocytes can become profoundly more acidotic than their surrounding milieu. The molecular mechanisms by which excessive acidosis might irreversibly injure astrocytes and, as a result, brain tissue are unknown. However, perhaps the most attractive hypotheses center on acid-

induced free radical injury (Pulsinelli et al., 1985; Siesjo et al., 1985).

Initially, indirect evidence was used to suggest that astrocytic intracellular pH (pH_i) can become 1 to 2 orders of magnitude more acidotic than the interstitial space and adjacent neurons (Kraig et al., 1985, 1986). Measurements of interstitial pH and tissue carbon dioxide tension (P_{CO_2}) were combined with gross estimates of intracellular volume distribution to suggest this preferential acidosis of astrocytes. Although these initial measurements have been the subject of dispute (Katsura et al., 1991, 1992), direct measurements of pH_i from presumed astrocytes are now available to resolve this controversy.

Intracellular, double-barrel, and pH-sensitive microelectrodes were used to determine the pH_i of astrocytes during terminal ischemia in rodents made hyperglycemic before the onset of terminal ischemia (Kraig and Chesler, 1990). Under these conditions two foci more acidic than interstitial space (6.17 to 6.20 pH) are found. The first is only occasionally seen at the onset of ischemia and reaches 3.82 to 4.89 pH, while the second is seen 5 to 46 minutes after the onset of ischemia and reaches 4.46 to 5.93 pH. A small direct current potential and massive increase in cell input resistance support the contention that these acidic foci are from intracellular space. Horseradish peroxidase injections via the reference barrels of selected pH-sensitive microelectrodes more obviously associate these extreme levels of acidosis with astrocytes. However, even the latter data must be regarded as preliminary, since astrocytic morphology changes so dramatically as the cells become extremely acidotic (Kraig and Chesler, 1990). Immunohistochemical characterization of dye-filled acidic foci, from work in progress, should confirm the identity of the acid foci as astrocytic.

Three principal events must occur for astrocytes to become more acidotic than their surrounding milieu during terminal ischemia, when all high-energy phosphates are depleted. First, continued acid accumulation must take place in these cells. Under anoxic conditions, protons are generated when adenosine triphosphate (ATP), made by anaerobic glycolysis, is hydrolyzed (Krebs, 1975). Brain glycolytic activity may be highest in astrocytes (Phelps, 1972; Passoneau and Crites, 1976). Furthermore, astrocytes are intimately involved in cellular and interstitial ion homeostasis, processes that are believed to require considerable ATP consumption (Walz, 1989). Thus, astrocytes may generate excessive acidity when they attempt to reestablish their ionic environments or that of the interstitial space (Hansen, 1985) after the onset of ischemia. The second requirement, that cellular membrane impermeability to ions remains intact, is discussed more extensively below. The third requirement for astrocytes to remain more acidotic than their outside environment is that more pH-related anions [e.g., lactate or bicarbonate (HCO_3^-)] remain outside these cells than are found within them (see Figure 64-1). Evidence already exists to support this notion of cation-anion distributions. For example, previous indirect measurements of pH and tissue P_{CO_2} show that more HCO_3^- is found in the interstitial space than in cells presumed to be astrocytes (Kraig et al., 1986). Furthermore, Walz and Mukerji (1988) have shown more lactate is found outside than within astrocytes in culture when these cells are made anoxic. However, experiments done in cultured systems need to be carefully evaluated when considered as evidence in support of observations made *in vivo*. Cells in culture are exposed to a relatively infinite external environment when compared to conditions *in vivo* (Hansen, 1985; Kraig et al., 1986; Smith et al., 1986). Indeed, such differences in experimental conditions may explain why the pH_i of astrocytes can rapidly equilibrate with the pH of the external milieu *in vitro* during various imposed pH perturbations (Mellergard et al., 1992), and yet remain an order of magnitude more acidic than the interstitial space *in vivo* during terminal ischemia (Kraig and Chesler, 1990).

The suggestion that at least one cell type, namely, astrocytes, can retain or increase their cell input resistance in the face of a loss of all high-energy phosphate stores goes against conventional logic of stroke pathophysiology. The usual assumption is that all brain cells lose the capacity to restrict ion movement across their plasma membranes with the loss of ATP stores. This belief probably arises from the following facts. First, ion gradients are largely determined by active processes (i.e., require ATP) (Erecinska and Silver, 1989). Second, high-energy phosphates are lost within a few minutes of the onset of global ischemia (Lowry et al., 1964). Third, large changes in interstitial ion concentrations that occur shortly after the onset of global ischemia suggest dissipation of these ion gradients (Hansen, 1985; Siesjo and Bengston, 1989). Fourth, ischemia causes the generalized release of neurotransmitters (Benveniste et al., 1984), which can increase membrane conductance to various ions. However, in spite of these strong implications, few direct measurements exist to confirm a total loss of membrane resistance during ischemia—indeed, evidence exists to the contrary.

Cell input resistance and plasma membrane resistivity are the best means by which to assess cell permeability to all ions. These passive electrical properties of cells are typically measured with intracellular

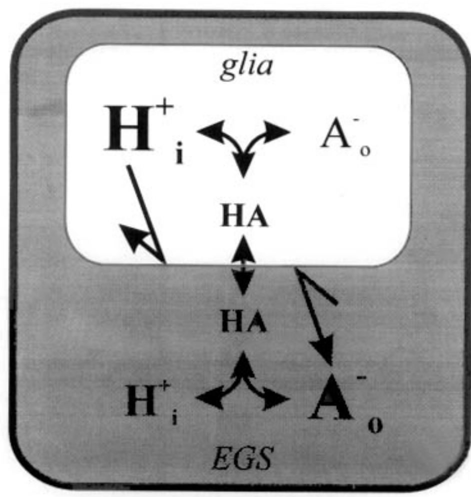


FIG. 64–1. Mechanism by which extreme astrocytic acidosis could be maintained during hyperglycemic and severe ischemia. The notion that a *trans*-membrane pH gradient could exist long after the loss of high-energy stores from brain ischemia is contrary to accepted general knowledge in stroke research. Most assume that membrane integrity is immediately lost with the loss of high-energy stores. Of course membranes will ultimately disintegrate from ischemia. However, data derived from cellular physiological techniques is now available to indicate that plasma membranes of astrocytes can remain intact for at least an hour after the onset of complete ischemia (see text for details). Direct measurements of presumed astrocytes during hyperglycemic and complete ischemia made with microelectrodes show that these cells remain more than an order of magnitude more acidotic than their surrounding milieu for up to an hour after the loss of high-energy phosphates from ischemia (Kraig and Chesler, 1990). The molecular mechanisms by which astrocytes accomplish this heretical feat remain undefined. Nonetheless, at least three essential phenomena must occur. First, astrocytic membranes must remain intact. Second, these cells must accumulate proton equivalents without absorbing bicarbonate from the interstitial space. Acidic inhibition of membrane transport systems for proton equivalents coupled to intact cell membranes and proton production within astrocytes would fulfill these requirements. Yet astrocytic membranes are still likely to remain permeable to nonionized organic acids (*HA*) such as lactic acid, so the concentration of HA would be expected to be equal within astrocytes and the extraglial space (*EGS*). Thus, a third requirement is needed for astrocytes to remain more acidic than the EGS. This requirement is simply that the EGS be a restricted compartment where the concentration of pH-related anions is greater than that found in astrocytes. If true, then the proton gradient across the astrocytic plasma membrane would simply be equal to the inverse of the *trans*-membrane anion ratio.

For example,

$$pH_i = pK_a + \log \frac{A_i^-}{HA_i} \quad (1)$$

and

$$pH_o = pK_a + \log \frac{A_o^-}{HA_o}, \quad (2)$$

where A_i^- and A_o^- are intracellular and interstitial conjugate bases, respectively (e.g., lactate or bicarbonate). If the ionization equilibrium constant (K_a) for a given acid is the same in the intracellular and interstitial space and that

$$[HA]_i = [HA]_o; \quad (3)$$

then by substituting Equations 1 and 2 into 3,

$$\frac{[H^+]_i}{[H^+]_o} = \frac{[A^-]_o}{[A^-]_o},$$

where H_i^- and H_o^+ are the intracellular and interstitial proton concentrations, respectively. [Modified from Kraig and Chesler (1990).]

or patch-clamp microelectrodes. Such measurements show that depolarization of neurons and glia are not associated with a total loss of cell input resistance. For example, neurons depolarized by exposure to hypoxia in brain slices show a dramatic reduction in cell input resistance (Fujiwara et al., 1987). However, they do not completely lose input resistance. In addition, neuronal input resistance only drops to about 40% of normal when these cells are depolarized by spreading depression (Czeh et al., 1992), a propagating phenomenon of susceptible brain tissue that has many metabolic and biophysical similarities to ischemia (Bures et al., 1974). Astrocytes lose even less of their normal input impedence during spreading depression. Most (Kraig, 1990) or all (Kraig, unpublished observations) of astrocytic input resistance is retained during spreading depression. The explanation for these observations must lie in studies of (1) ion channel, receptor, and transporter density as well as distribution in brain cells; (2) the combined effects of changes in intracellular and interstitial milieu on these membrane-based mechanisms for ion translocation; and (3) retained integrity of the plasma membrane lipid bilayer. Perhaps even more startling than preserved ion impermeability during depolarization is the realization that cell input resis-

tance of astrocytes can *rise* when ATP stores are exhausted. When cultured astrocytes are poisoned by blockade of aerobic and anaerobic glucose metabolism their membrane potential declines to near zero (Harold and Walz, 1992). However, under these conditions, the input resistance of these cells does not fall but instead rises by more than an order of magnitude!—precisely what is seen *in vivo* during hyperglycemic terminal ischemia (Kraig and Chesler, 1990).

How astrocytic input resistance could rise is unknown but can be attributed to changes in plasma membrane surface area, specific resistance of the cell membrane, and/or the degree of electrical coupling between adjacent glia cells. Acidosis can influence each of these variables. First, morphological evidence, obtained from horseradish peroxidase injections, suggests distal processes of astrocytes are lost when these cells become extremely acidotic (Kraig and Chesler, 1990). Loss of astrocytic endfoot processes (during global ischemia) was first recognized by Alzheimer in 1910 (Penfield, 1928). He noted that astrocytes can undergo a process he termed "clasmatodendrosis" (i.e., disintegration of branches), which results in these cells assuming an ameboid shape with a clear reduction in surface area. Since most of the potassium conductance of astrocytes may be in their endfoot processes (Newman, 1986), loss of these distal processes in ischemia would be expected to result in a rise in cell input resistance. Second, the specific membrane resistance of astrocytes may rise with acidosis, since conductance through potassium-sensitive channels decreases with a reduction of extracellular pH (Walz and Hinks, 1987). Indeed, ion channels show an increase in open probability with alkalinization and a pronounced decreased below pH 7 (Lang et al., 1988). Third, astrocytic coupling via gap junctions falls during hypercapnia (Conners et al., 1984) or exposure to lactic acid (Anders, 1988) and therefore, presumably during acidosis from ischemia.

Whether these astrocytic changes, namely, extreme acidity ($\approx$5.3 pH), increased input resistance, and loss of distal processes influence the evolution of infarction remains unknown. One must first note that these three fundamental changes in ischemic astrocytes have only been defined, thus far, during hyperglycemic and terminal ischemia. Reperfusion is needed for the development of infarction (Graham, 1992). Preliminary evidence (Kraig, unpublished observations) suggests that astrocytes also become more acidotic (e.g., $<$6 pH) than their external environment (6.2 pH) during severe global ischemia under hyperglycemia (e.g., blood glucose $\approx$400 mg/dl), conditions known to produce infarction (Pulsinelli et al., 1982b). Furthermore, when lactic acid is injected directly into brain for a period equal to that which can produce infarction from global ischemia (and hyperglycemia), a threshold pH of 5.3 must be surpassed to cause tissue necrosis (Kraig et al., 1987).

How astrocytic pH_i changes during focal ischemia is unknown. However, several lines of evidence suggest that astrocytic pH_i can be an order of magnitude higher in focal than global infarction, and yet in both instances, infarction can still develop from a common pH-related mechanism (Kraig, 1990). This suggestion follows from the following considerations of astrocytic HCO_3^-. First, during hyperglycemic and terminal ischemia, astrocytic pH_i reaches 5.3 (Kraig and Chesler, 1990), while tissue P_{CO_2} is approximately 400 torr (Kraig et al., 1986; Katsura et al., 1992). Assuming tissue P_{CO_2} is at least a minimum estimate of astrocytic CO_2, the HCO_3^- concentration of these cells can be calculated via the Henderson-Hasselbach equation to be approximately 2 mM (Kraig and Chesler, 1990). Bicarbonate is essential for the sequestration of iron within transferrin. Free iron in an excessively acidic environment (e.g., 2 mM HCO_3^-?) may catalyze the generation of free radicals with subsequent irreversible injury of membranes by lipid peroxidation (Pulsinelli et al., 1985; Siesjo et al., 1985). If the same injurious process proceeds within astrocytes during focal ischemia, the increased level of blood flow found there compared to global ischemia would mean tissue P_{CO_2} and (CO_2 within astrocytes) was lower than 389 torr, perhaps about 50 to 100 torr. This would mean astrocytic pH_i would be 5.9 to 6.2 if the critical level of HCO_3^- was also 2 mM.

It seems unlikely that all astrocytes uniformly will surpass some critical reduction in HCO_3^- during the development of infarction, since residual blood flows through ischemic zones in global (Pulsinelli et al., 1982a) and focal (Ginsberg, 1990) ischemia. Residual blood flow could allow continued glucose delivery to brain and escape of proton equivalents. Thus, it is likely that the pH_i of astrocytes will vary in space and time, depending on unique environmental conditions of individual cells. Support for this suggestion can be seen even in terminal ischemia, where no blood flows through ischemic brain, yet average pH_i is heterogeneously distributed throughout cortex (LaManna et al., 1992). With blood continuing to flow residually through ischemic tissue, such a pH_i heterogeneity might only be enhanced. This implies that if pH (or astrocytic pH_i) is a sole determinate of infarction, then infarction will proceed from separated microfoci that coalesce with time. Alternatively, pH may not be the sole determinant of infarction. Instead, other cellular changes might also

result in infarction or perhaps be responsible for the coalescence of acid-induced microinfarcts.

Another cellular change that either could be injurious or amplify the affect of changes in pH_i is alterations in intracellular calcium ($Ca^{2+}{}_i$). This possibility arises from observations that cultured astrocytes can be injured irreversibly by transient exposure to reduced levels of extracellular Ca^{2+} or reversibly by prolonged exposure to excitatory amino acids.

The "Ca^{2+} paradox," first described in heart, occurs after a transient period of reduced extracellular Ca^{2+}. When the Ca^{2+} concentration of the perfusate is returned to normal, myocardial Ca^{2+} rises from a large influx (Alto and Dhalla, 1979). Young believes the Ca^{2+} paradox may be important to the pathogenesis of ischemic and traumatic brain injury (Young, 1986, 1987). When neural cells (astrocytoma cells, spinal glia, and neuroblastoma cells) are exposed to a transient period of reduced extracellular Ca^{2+}, $Ca^{2+}{}_i$ rises only in glial cells after the incubation media is returned to one with a normal level of Ca^{2+} (Kim-Lee et al., 1992). Furthermore, phase contrast microscopy shows that only glia are irreversibly injured by this process. If such a process occurs *in vivo*, perhaps perineuronal astrocytes are selectively killed after ischemia, and their death then results in the loss of selectively vulnerable neurons (Kim-Lee et al., 1992).

Astrocytes may also be injured in a Ca^{2+}-dependent fashion following exposure to excitatory amino acids released during ischemia (Koyama et al., 1991). In culture, astrocytes swell and their nuclei become pale and watery after several hours incubation with L-glutamate, L-aspartate, or L-alpha-amino diacids such as L-homocysteic acid (Bridges et al., 1992). Although the swelling induced by L-alpha-amino diacids eventually leads to cell lysis, that produced by glutamate and aspartate appears ultimately reversible. The mechanisms underlying astrocytic swelling and recovery *in vitro* or *in vivo* remain only partially defined, and how Ca^{2+} might influence astrocytic volume regulation is even less clear. Moreover, the functional consequences of cell swelling, short- and long-term, both to the astrocyte and to the surrounding brain during ischemia are unclear. It is possible that astrocytic mechanisms for ion and neurotransmitter homeostasis become disrupted during swelling, producing the aberrations in interstitial K^+, pH and neurotransmitters that ultimately lead to cell death.

Of course other changes associated with disturbances in pH_i and $Ca^{2+}{}_i$ homeostasis described above may also contribute to effect injury of brain cells. Astrocytes, for example, have long been thought to be the principal regulators of interstitial K^+ homeostasis through K^+ uptake and spatial buffering (Barres, 1991). The excessively high interstitial K^+ concentrations seen in ischemia could potentially arise from changes in pH_i and $Ca^{2+}{}_i$ directly influencing the mechanisms underlying K^+ homeostasis. As discussed below, alkalotic shifts in astroglia during evoked neuronal activity or spreading depression are associated with a simultaneous rise in intracellular concentrations of HCO_3^- and K^+ (Kraig and Chesler, 1988). If HCO_3^- serves as a requisite anion for K^+ uptake, its depletion from acid production during ischemia would greatly impede astrocytic removal of K^+ from the interstitial space. In addition H^+ ions modulate the activity of various membrane channels (Kimelberg, 1988; Lang et al., 1988; Gifford et al., 1990; Tang et al., 1990; Vyklicky et al., 1990; Kaku et al., 1993) and transporters such as the Na/K ATPase (Breitwieser, 1987); acidosis might effectively shut these pathways for K^+ entry into astrocytes during ischemia. Finally, high astrocytic $Ca^{2+}{}_i$, such as can occur from exposure of these cells to excitatory amino acids (Finkbeiner, 1993) or activation of voltage-sensitive Ca^{2+} channels (Barres et al., 1989), could lead to the release of K^+ from astrocytes via Ca^{2+}-activated K^+ channels (MacVicar et al., 1991), if astrocytes were first depolarized past their equilibrium potential for K^+. In kainate-lesioned brain slices and in culture, astrocytes are known to release K^+ in response to kainate (MacVicar, 1988).

H^+ or Ca^{2+} ions alone are probably rarely the sole determinants of cell death from ischemia. Most parameters of cell vitality, when taken past some extreme, may become a factor critical to the destruction of cells. In this regard severe brain (and perhaps astrocytic) acidosis may be the most important factor involved in infarction from global ischemia under hyperglycemic conditions. However, as ischemic conditions become less absolute (i.e., as in focal ischemia) it is likely that other key variables will interact with pH changes to effect cell injury. It is plausible that pH changes may combine with other phenomena to protect cells from injury.

ASTROCYTES MAY AMELIORATE ISCHEMIC BRAIN INJURY

The notion that increased acidity in ischemic brain could enhance neuronal survival initially came from evidence that showed glutamate (e.g., *N*-methyl-D-aspartate) receptor-mediated whole-cell currents in neurons are reduced at acidic extracellular pH (Tang et al., 1990; Traynelis and Cull-Candy, 1990; Vyk-

licky et al., 1990). Furthermore, lowered extracellular pH reduces injury of neurons *in vitro* from glutamate exposure (Gifford et al., 1990; Tombaugh and Sapolsky, 1990; Kaku et al., 1993). However, in light of recent work from Siesjo's laboratory it is uncertain if these results are relevant to conditions *in vivo*. Katsura and coworkers (1994) show that acidosis from hypercapnia aggravates neuronal injury from global ischemia instead of reducing it. Taken together, these results suggest that acidity from ischemia (at least of the interstitial space) may under certain circumstances protect brain tissue from irreversible injury. Yet if such protection can occur from increased acidity *in vivo*, it is likely to do so from events that are more complex than a simple modulation of ionic conductances.

Given that astrocytes are intimately involved in both interstitial pH regulation (Walz, 1989) and brain glutamate homeostasis (Schousboe et al., 1993), these cells might enhance the survival of ischemic neurons. Evidence for this suggestion has been derived from experiments with cultured cells. For example, astrocytes increase the survival of cultured neurons to transient exposure to hypoxia (Yu et al., 1989) or anoxia (Vibulsreth et al., 1987). Furthermore, neuronal cultures are less susceptible to the excitotoxic effects of glutamate when astrocytes are also present (Rosenberg and Aizenman, 1989; Sugiyama et al., 1989). Whether or not this effect is solely due to alterations in glutamate homeostasis remains controversial (Vibulsreth et al., 1987). Elevated glucose levels can also protect astrocytes in culture from irreversible injury by anoxia (Kelleher et al., 1993). As mentioned previously, caution should be exercised when extrapolating these *in vitro* observations to those anticipated *in vivo* because of (1) the relatively infinite extracellular milieu of culture systems compared to the interstitial space *in vivo*; (2) the tendency to keep extracellular constituents at normal levels and only alter experimental variables *in vitro*, rather than change extracellular constituents to levels known to exist during ischemia (e.g., reduced levels of sodium, chloride, calcium, bicarbonate, and phosphate, and elevated levels of protons, carbon dioxide, and potassium) in parallel with changes of experimental variables; (3) the tendency to use primary culture systems *in vitro*, which preclude subtle (or as yet undefined) cell-cell interactions that would otherwise be present. With these caveats aside it should be said that if pH (and/or astrocytes) can be both harmful or helpful, it will be because of the way this physicochemical variable and cell type interact with other relevant variables and cell types. If true, this suggests that experiments designed to examine the cellular (and potentially, molecular) bases for ischemic brain injury should strive to study these phenomena in tissue-based structures with tools that have the capacity to make measurements at a cellular level.

REACTIVE GLIOSIS

Astrocytic Changes

If astrocytes are not destroyed along with other brain cells by ischemia (e.g., infarction) they are transformed into so-called reactive species (Graham, 1992). Reactive astrocytosis is defined as a hypertrophy and hyperplasia of these cells that is associated with increased glial fibrillary acidic protein (GFAP) (Graham, 1992). This classical denotation has been extended by Petito and coworkers (1990) who have shown that global ischemia can induce two variations of reactive astrocytosis. The first consists of hypertrophy, increased GFAP staining, and hyperplasia that persists for at least 5 weeks after ischemia. It is seen in zones where neurons are destroyed. The second consists of astrocytic hypertrophy and increased GFAP staining, which persists for about 2 weeks and is not associated with astrocytic hyperplasia. The latter is seen in neocortex where few neurons are destroyed.

How astrocytes are transformed into reactive species remains uncertain. Spreading depression (SD), a gross perturbation of brain, can be used to study the phenomenon of reactive astrocytosis. SD is defined as a propagating, transient loss of spontaneous and evoked neuronal activity that is associated with a large, negative direct current potential in interstitial space (Bures et al., 1974). It is known to occur *in vivo* as well as *in vitro* in brain slices (Snow et al., 1983) and, potentially, explant cultures (Dani et al., 1992) where normal brain cytoarchitecture largely remains intact (Gähwiler et al., 1992). SD has many metabolic and biophysical similarities to processes that occur during ischemia (Bures et al., 1974). Large changes of interstitial potassium, sodium, hydrogen, calcium, chloride (Nicholson and Kraig, 1981), HCO_3 and carbon dioxide (Kraig and Cooper, 1987), glutamate, and lactate (Krivanek, 1961; Mutch and Hansen, 1984; Scheller et al., 1992) as well as in arachidonic acid (Lauritzen et al., 1990) occur. Importantly, however, SD does not cause irreversible injury of brain cells (Nedergaard and Hansen, 1987) and does not open the blood-brain barrier (de-Araujo-Pinheiro and Martins-Ferreira, 1984).

SD causes changes in astrocytes that resemble the reactive astrocytosis described by Petito and col-

leagues in neocortex after global ischemia (Kraig et al., 1991). Recurrent SD induces a significant increase in GFAP staining and astrocytic hypertrophy that persists for 2 weeks before returning toward normal by 4 weeks. This increase in GFAP staining is associated with a parallel, significant increase in GFAP content that seems mostly due to cellular hypertrophy and not hyperplasia (Kraig et al., 1995).

Although the mechanisms by which astrocytes are transformed into reactive species following SD remain unknown, changes in the concentration of various ionic species must be considered as prime candidates. For example, a rise in pH_i is commonly associated with increased metabolic activity (Busa and Nucitelli, 1984). During SD, as the brain generates lactate (Krivanek, 1961; Mutch and Hansen, 1984; Scheller et al., 1992) and the interstitial pH principally swings more acid (Kraig et al., 1983; Mutch and Hansen, 1984), astrocytes become markedly more alkaline. Astrocytic pH_i is normally around 7.0. During SD it typically rises to 7.6 to 7.8 before returning to normal as the cells repolarize (Chesler and Kraig, 1987, 1989) (Figure 64-2). A depolarization-induced alkalinization occurs in a variety of cells and has recently been characterized in cultured astrocytes (Boyarsky et al., 1993). Others have argued that a rise in pH_i is not essential for the initiation of anabolic activities (Ganz et al., 1989). The latter view, however, is tempered by the suggestion that a rise in pH_i is necessary only in those cells whose resting pH_i is not already sufficiently alkaline (Whitfield, 1990). An alternative possibility to pH_i changes is that a rise in intracellular potassium initiates reactive astrocytosis (Kraig and Jaeger, 1990). The massive rise in pH_i described above is associated with an approximate 50 mM increase in HCO_3^- concentration (Kraig and Chesler, 1988) that is probably accompanied by a nearly equivalent rise in intracellular potassium (Kraig and Jaeger, 1990). 3T3 fibroblasts divide when their internal potassium rises above 100 mM, a change seen in astrocytes during SD (Lopez-Rivas et al., 1982). Glutamate, which also depolarizes astrocytes (Bowman and Kimelberg, 1984), may initiate reactive astrocytosis, perhaps because of a rise in $Ca^{2+}{}_i$ (Cornell-Bell et al., 1990; Glaum et al., 1990; Jensen and Chiu, 1990) and such a rise is a well-recognized trigger for increased vital processes within cells (Busa and Nucitelli, 1984; Whitfield, 1990). Astrocytic $Ca^{2+}{}_i$ is known to rise during SD (Kraig and Lascola, 1992, 1994) (Figure 64-3), and thus this rise may be stimulated through neurotransmitter receptors such as ionotropic and

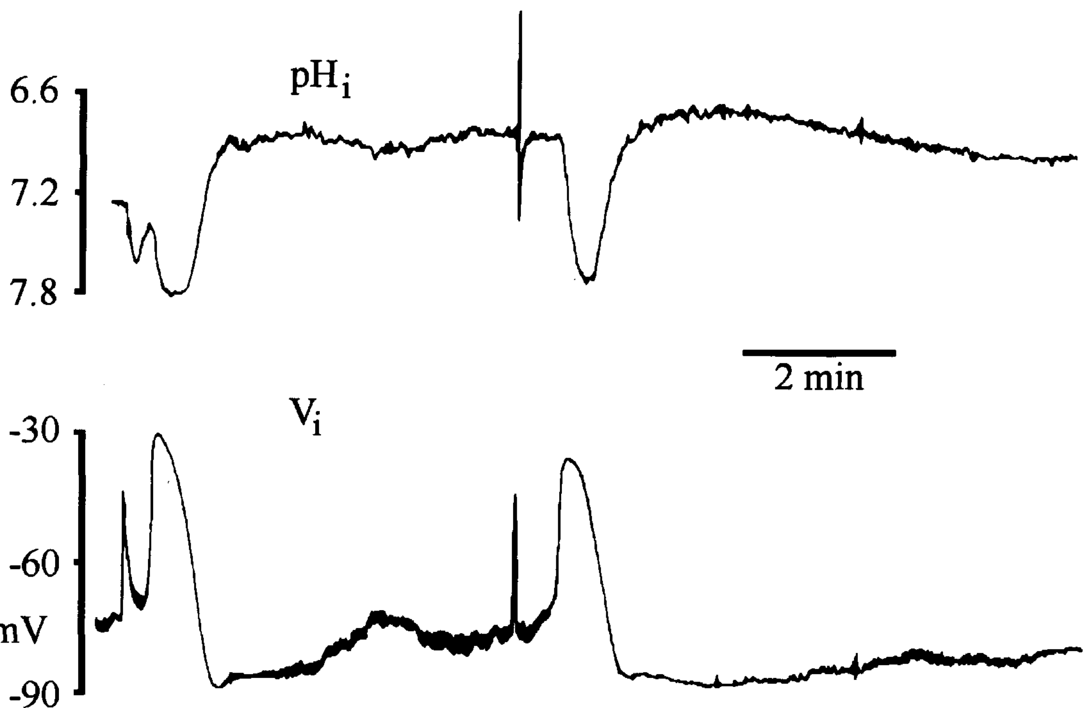


FIG. 64–2. Astrocytic pH_i responses to successive spreading depressions (SDs). Records show pH_i (*upper record*) and simultaneously measured membrane potential (V_i) (*lower record*) from a rat neocortical astrocyte measured *in vivo* with a double-barrel pH-sensitive ion-selective microelectrode. SD was induced by brief 100 Hz surface, bipolar electrical stimulation (*arrows*). Resting astrocytic pH_i is 7.0 and during SD can rise to as much as 7.6 to 7.8 before swinging slightly acid during repolarization and recovery from SD. (Modified from Chesler and Kraig (1989).]

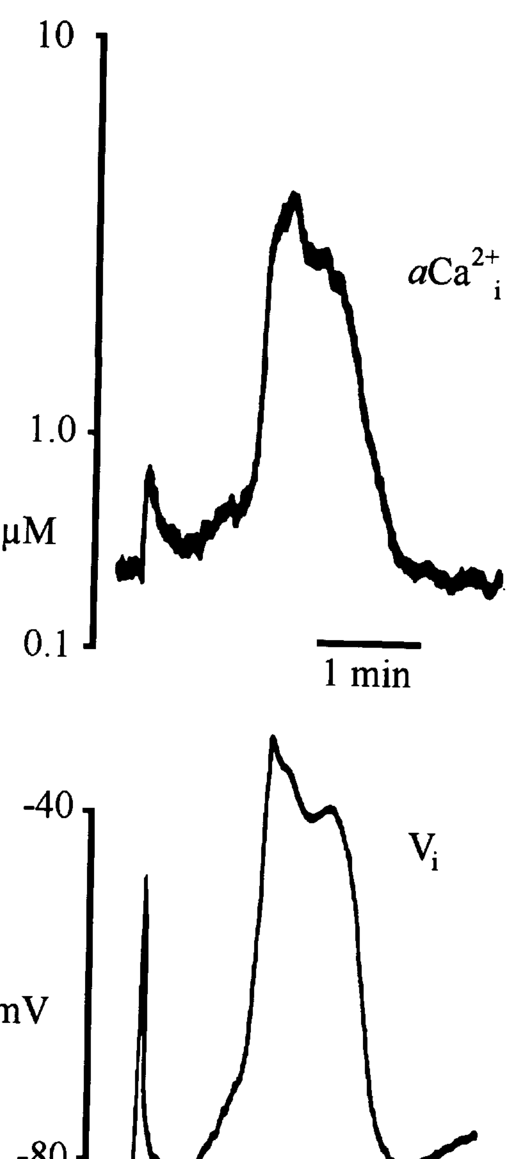


FIG. 64–3. Astrocytic $Ca^{2+}{}_i$ change during spreading depression. Astrocytic $[Ca^{2+}]_i$ was measured with a Ca^{2+}-sensitive and double-barrel microelectrode. A 100-Hz surface, bipolar electrical stimulus was used to elicit SD (*arrow*). Astrocytic $Ca^{2+}{}_i$ activity (*upper record*) rose from a baseline of about 200 nM to more than 3 μM during maximal depolarization as shown by simultaneous recording of membrane potential (*lower record*). Membrane potential was initially -82 mV before falling to -30 mV during SD. [Modified from Kraig and Lascola (1994).]

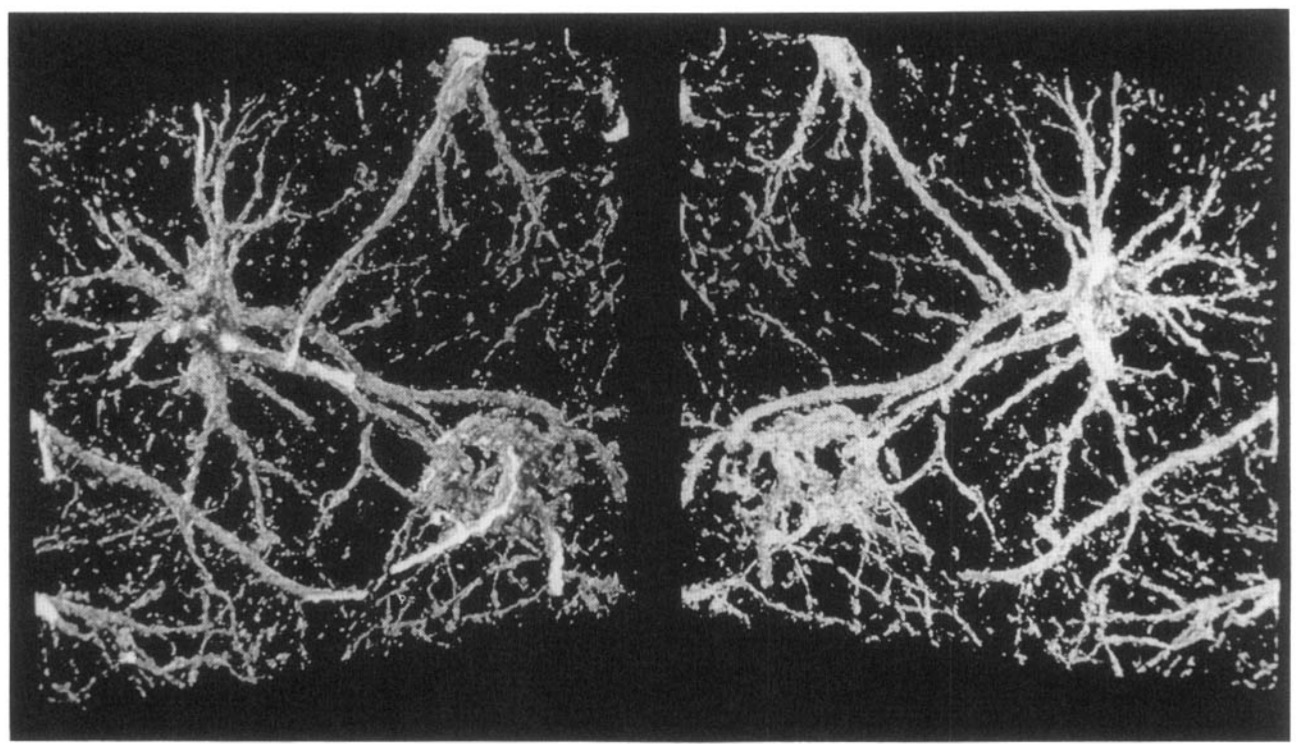
metabotropic glutamate channels believed to present on astrocytes.

It is possible that reactive astrocytosis is not triggered by any singular change in some variable but instead reflects the combined effects of various changes. An example of this possibility is the potential synergistic effect of changes in pH_i and $Ca^{2+}{}_i$. Busa and Nucitelli (1984) were the first to point out that a rise in pH_i might affect the degree to which a rise in $Ca^{2+}{}_i$ influences calcium-dependent processes. They reasoned that a rise in pH_i of 0.5 would have the same effect as a fivefold rise in $Ca^{2+}{}_i$. Astrocytic pH_i rises by more than 0.5 during SD and $Ca^{2+}{}_i$ rises by 1 or 2 orders of magnitude. Together these ionic changes may be powerful synergists for astrocytic activation.

Cultured astrocytes are often used to examine how astrocytes are transformed into reactive species (for review as Eddleston and Mucke, 1993). An important caveat with the use of cultured systems for studying this phenomenon ought to be mentioned. Astrocytes in culture never fully mature. They continue to express vimentin, for example, while only developing astrocytes or ones made reactive do so *in vivo* (DeVellis et al., 1986). Thus, cultured astrocytes may already be "reactive" in at least some aspects of their behavior. Nonetheless, isolated systems have distinct experimental advantages over whole-animal preparations. To explain the mechanisms by which ischemia transforms astrocytes into reactive species will, no doubt, require the association of studies done both *in vitro* and *in vivo*. It is our belief that the utility of such studies can be maximized when they are done in parallel so that analogies can easily be drawn between these two systems.

Microglial Changes

Not only ionic species but *cells* may interact to effect astrocytic changes. After ischemia, microglial cells respond by changing their morphology, dividing and increasing the expression of major histocompatibility complex (MHC) antigens (Finsen et al., 1993). Microglia secrete and are influenced by a host of cytokines and their activation may allow these cells to induce reactive changes in astrocytes (Benveniste, 1992). This has led some to wonder if an ischemia-induced increased permeability of the blood-brain barrier allows soluble cytokines from the vasculature to affect changes in microglia (Finsen et al., 1993). Alternatively, stimuli that activate microglia cells may come from within the brain. For example, Gehrmann and coworkers (1993) speculate that the unique pattern of potassium channels found on microglia *in vitro*, if present *in vivo*, would make these cells highly susceptible to depolarization by potassium released from neurons. They sought to test this possibility by determining if SD could activate microglia *in vivo*. They show that after 1 hour of recurrent SD, microglia are activated (e.g., increased OX-42, OX-18 (MHC-I) and OX-6 (MHC-II) immunohistochemical staining) (Gehrmann et al., 1993). (OX-42 is an antibody that recognizes the



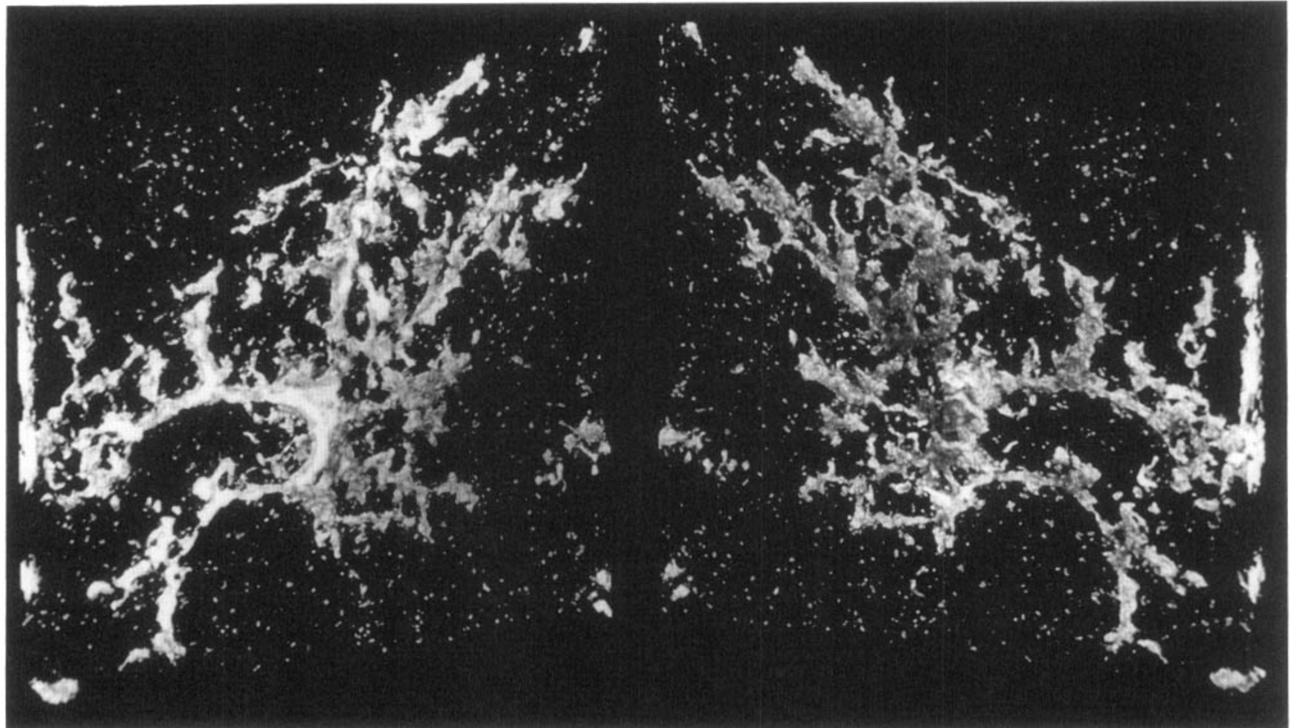


FIG. 64-4. Three-dimensional reconstructions of deconvolved glial images produced by computer-based digital confocal microscopy. Brightfield images of reactive astrocytes (*upper panel*) and normal microglia (*lower panel*) are shown. Astrocytes were stained with GFAP and microglia with OX-42. Both staining protocols used diaminobenzidine as the chromogen. Images were created with a 12-bit resolution cooled CCD camera (CH250; Photometrics, Inc.) through a red (660 nM) filter. Images were deconvolved and reconstructed using Microtome and Voxblast, respectively, two software packages available from Vaytek, Inc. Resultant images were false-colored, turned into photographic slides and finally into prints. Left: Front views. Right: Back views of the same cells. These images begin to illustrate the fine detail and three-dimensional aspects of brain cells that can be seen using digital confocal microscopy. (Kraig, Caggiano and Lascola, unpublished observations.)

complement receptor-3 surface adhesion molecule on microglia). The activation of microglia from SD is transient, reaching a peak at 24 hours after SD before returning to normal in approximately 3 days. Since these workers could elicit a microglial and not astrocytic activation after 1 hour of SD, they concluded that microglial activation might precede (and be more easily elicited?) than that of astrocytes. Indeed, Gehrmann and coworkers elicited an average of 8 SDs over an hour, while work from our laboratory has used a paradigm of 3 hours of SD (and an average of 21 SDs) to activate microglia (Caggiano and Kraig, 1993) and astrocytes (Kraig et al., 1991). Thus, the possibility that SD activates microglia earlier than it does astrocytes or even that SD can activate microglia preferentially remains an exciting possibility.

What causes the activation of microglia from ischemia remains unknown. Blood-brain barrier permeability is not increased during SD (de-Araujo-Pinheiro and Martins-Ferreira, 1984). Thus, if activation from SD proceeds by the same mechanisms that occur during activation from ischemia, disruption of the blood-brain barrier does not seem to be a necessary prerequisite. Not only do microenvironmental signals endogenous to brain (Gehrmann et al., 1993) need to be considered as potential stimuli for activation of microglia (and astrocytes) but also systemic signals. For example, systemic administration of tumor necrosis factor-α or interfon-γ lead to increased MHC-II expression on microglia without causing tissue injury (Vass and Lassmann, 1990; Lassmann et al., 1991). Furthermore, endocrine-immune interactions can modulate reactive gliosis. Systemic administration of steroids significantly reduces not only microglial but astrocytic gliosis from SD (Caggiano and Kraig, 1993). Clearly, the behavior of microglia and that of astrocytes seems destined to be defined as an intimate interaction between changes within and outside of brain.

SUMMARY AND CONCLUSIONS

Glial cells, like other living things, do not exist in isolation. Rather they interact with other brain cells, the vasculature and substances from other organ systems. The concept that interactions necessarily occur between glia and their environment is likely also to apply to how these cells respond to ischemic brain injury. The notion of interaction may be applied equally well to how ions, molecules, cells, and even tissues influence the behavior of these cells.

Given the high capacity of astrocytes (and microglial) to influence and be influenced by changes in their microenvironment, one must decide how best to study reactive gliosis. Animal preparations that include multiple cell types seem best suited for the examination of interactive cellular behavior. Whole-animal studies are indispensible for the definition of the magnitude and extent of changes that occur in experimental variables during and after ischemia. However, whole-animal studies are limited by a relative inability to manipulate experimental conditions compared to *in vitro* preparations. Improved access is provided by *in vitro* preparations but with the advantage of "complete control" of the external milieu, comes the responsibility for defining all aspects of this control. Brain slices are well suited for acute studies that examine plasma membrane-based issues of cellular interaction, while mixed and cocultures are well suited for more long-term studies. Reaggregate and slice culture systems may be the most advantageous tissue culture preparations, since a high degree of cytoarchitectural integrity is maintained in these systems. Techniques most useful for the assessment of reactive gliosis include those that emphasize the use of micropipettes and microscopy. The former include intra- and extracellular electrodes, patch-clamp pipettes and ion-selective microelectrodes, while the latter include video microscopy and quantitative voltage and ion-selective dyes as well as immunohistochemical imaging that can be coupled to a confocal microscope (Figure 64-4). These preparations and techniques are those that seem likely to maximize astrocytic (and microglial) abilities to interact with their external environment.

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65 Idiopathic and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinsonism

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The precise mechanisms of neuronal degeneration in human neurologic disorders remain mostly unknown. In the past few years, however, studies utilizing novel or refined experimental models have suggested that nerve cell death may be the result of a concerted sequence of metabolic, biochemical, and toxic events. Current knowledge also suggests that, although the final target of neurodegeneration may be a rather selective nerve cell population, other types of cells may play a role in degenerative processes. In recent years, glial elements have attracted increasing attention as possible participants in the pathogenesis of neuronal damage.

Parkinson's disease is one of the most common neurodegenerative disorders of aging; it results from the selective loss of dopaminergic neurons in the nigrostriatal pathway. It is an excellent example of how our knowledge about neurodegenerative processes has been enhanced and reshaped in the past few years thanks to the availability of new experimental tools. Ten years ago it was discovered that a simple pyridine derivative, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), was able to cause the selective destruction of nigrostriatal dopaminergic neurons (Burns et al., 1983; Langston et al., 1984a) and a syndrome in humans that was clinically indistinguishable from idiopathic parkinsonism (Langston et al., 1983). The MPTP model of parkinsonism has since provided a number of clues about the mechanisms that might lead to neurodegeneration in Parkinson's disease as well as other neurologic disorders. Of particular interest are the results from studies of MPTP-induced neurotoxicity which support the hypothesis that a multistep process leads to neuronal death; these studies have also suggested that an active role is played by astrocytes. In this chapter, the mechanisms of neuronal death in idiopathic and MPTP-induced parkinsonism will be discussed. Events that are likely to involve astrocytes are emphasized so that the reader can expect to obtain insight into the direction of future studies concerning the role of glial cells in neurodegenerative disorders.

PARKINSON'S DISEASE

Clinical and Pathological Features

Idiopathic Parkinson's disease is a progressive age-related neurodegenerative disorder. It is rarely observed before the age of 40 and its prevalence increases with advancing age (Kurland et al., 1973). Its clinical manifestations are essential for the diagnosis, due to the absence of measurable biological markers of the disease; indeed, the clinical diagnosis of Parkinson's disease can only be confirmed postmortem by pathologic examination of the brain. Cardinal features of Parkinson's disease include bradykinesia, resting tremor, rigidity and postural instability. Once the diagnosis is made, the clinical syndrome advances usually at a relatively slow rate, progressively impairing, however, the normal activity of the patient and his ability to care for himself.

Degeneration of the pigmented neurons in the pars compacta of the substantia nigra represents the cardinal neuropathologic feature of Parkinson's disease. Nerve cell loss is not equally severe throughout the substantia nigra and it is not confined within this region of the brain, however. The most severe cell loss is observed in the lateral and ventral cell groups of the substantia nigra (Gibb et al., 1990), and neuromelanin-containing noradrenergic neurons of the locus ceruleus are also affected by degenerative processes in nearly all typical cases of Parkinson's disease (Forno, 1982). Another hallmark of neuronal degeneration in Parkinson's disease is the presence of eosinophilic inclusions, called Lewy bodies, within the cytoplasm of nerve cells (Forno, 1982). The significance of Lewy bodies remains unknown, although they are thought to be indicative of an ongoing degenerative process. The most common ob-

servation concerning astrocytes in the parkinsonian brain is the presence of glial scars; they are usually localized in the areas most severely affected by neurodegeneration (*i.e.*, the lateral and ventral portions of the zona compacta of the substantia nigra) and are most likely the result of a secondary reaction to the nerve cell loss (Forno et al., 1992).

A large number of reactive microglia phagocytosing dopaminergic cells have been observed in the substantia nigra pars compacta of parkinsonian brains using immunohistochemical staining for HLA-DR (McGeer et al., 1988). HLA-DR is a human glycoprotein of the group II major histocompatibility (MHC) class; its major function is probably to present foreign antigen to T-helper lymphocytes. The precise role of HLA-DR-positive microglia in dopaminergic cell death remains to be clarified. However, the finding by McGeer and colleagues is consistent with the hypothesis that an ongoing neuropathological process underlies Parkinson's disease and is still active at the time of death of parkinsonian patients.

Neurochemistry

From the neurochemical point of view, Parkinson's disease is characterized by a severe depletion of dopamine in the nigrostriatal pathway. This depletion is the result of death of the dopaminergic neurons which are localized in the substantia nigra and project their axons to the striatum (Anden et al., 1984). Similar to the unequal distribution of neurodegeneration in the substantia nigra, a gradient of dopamine loss is observed in the striatum, with a greater depletion in the putamen than in the caudate; also, within the putamen, the posterior region is more affected than the anterior portion (Kish et al., 1988).

As already mentioned, the pathogenesis of neuronal death in Parkinson's disease is far from being understood. Among the numerous hypothesized mechanisms, the one that has received the most attention in the past decade is perhaps the oxidative stress hypothesis (Cohen, 1983). Oxidative stress is defined as a toxic condition in which the cellular oxidation/reduction (redox) balance is shifted toward oxidation. This may occur as a consequence of accumulation of oxygen free radicals (*e.g.*, the superoxide anion radical, O_2^-, or hydrogen peroxide, H_2O_2) due to an excessive production and/or decreased scavenging ability; oxygen radicals are very reactive chemical species able to oxidize DNA, membrane lipids, essential enzymes, and structural proteins, thus causing impaired cellular activity and eventually cell death.

It has been suggested that the brain in general may be particularly susceptible to free radical damage for reasons such as the high content in polyunsaturated fatty acid side chains of the membrane lipids and the relative poor activities of enzymatic defense mechanisms (*e.g.*, catalase, superoxide dismutase, and glutathione peroxidase). There are also reasons to hypothesize that, within the brain, the monoaminergic system may represent a preferential target for oxidative stress. Both the enzymatic and nonenzymatic oxidation of dopamine can generate H_2O_2:H_2O_2 is a product of dopamine conversion via monoamine oxidase (MAO) and could also be formed during dopamine autoxidation (Graham, 1978) (Figure 65–1). Under normal conditions, H_2O_2 is reduced enzymatically by catalase and glutathione peroxidase. However, if the rate of H_2O_2 production overwhelms these defense systems, H_2O_2 could be accumulated, resulting in the oxidation of essential cellular macromolecules. Furthermore, in the presence of transition metals such as iron and copper, H_2O_2 could generate even more reactive and deleterious oxygen species,

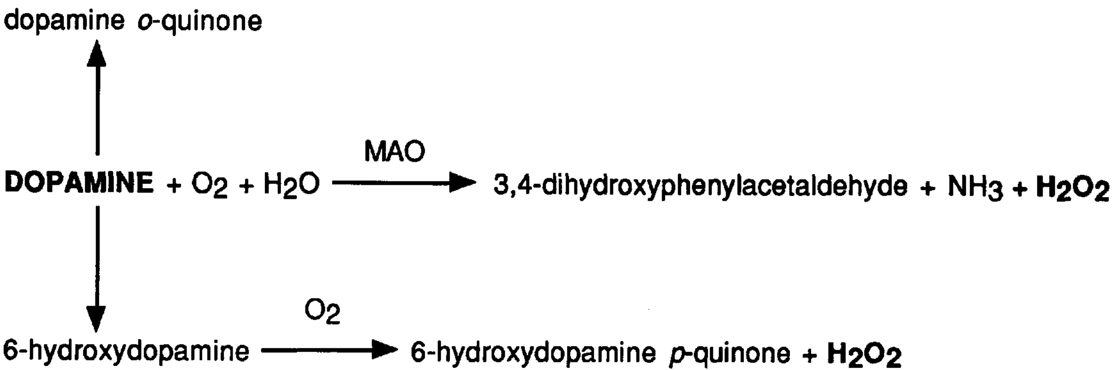

FIG. 65-1. Oxidative pathways of dopamine metabolism. Hydrogen peroxide (H_2O_2) could be formed during dopamine autoxidation and as a product of dopamine metabolism via monoamine oxidase.

such as the hydroxyl radical (Halliwell and Gutteridge, 1984). Interestingly, iron is unevenly distributed throughout the brain (Hill, 1988), and the relatively high content of iron in the substantia nigra may represent another mechanism for an increased susceptibility of nigrostriatal dopaminergic neurons to oxidative stress. The substantia nigra is also rich in neuromelanin, a product of catecholamine autoxidation (Graham, 1978). Neuromelanin can bind iron (Youdim et al., 1989), and reactions involving neuromelanin could represent additional sources for free radicals.

If dopamine metabolism and oxygen radical production play a role in the nigrostriatal nerve degeneration in Parkinson's disease, then it is conceivable that astrocytes may be involved in these processes. The relationships between glial cells, dopamine turnover, oxidative stress, and loss of dopaminergic activity in Parkinson's disease are schematized in Figure 65-2 and are discussed in the following section.

Role of Glial Cells in Dopamine Metabolism and Oxidative Stress

The action of dopamine as a neurotransmitter is terminated at least in part by its extraneuronal metabolism, and a key role in this metabolism is attributed to MAO-B, an enzyme localized within astrocytes (Levitt et al., 1982; Westlund et al., 1985). For these reasons, changes in glial MAO-B have been suggested to affect dopaminergic neurotransmission (Knoll, 1985) and may ultimately contribute to the main pharmacologic feature of Parkinson's disease, that is, the decline of nigrostriatal dopaminergic activity. Interestingly, significant changes in MAO-B do occur physiologically in the brain: higher MAO-B levels have been measured in older humans and older animals and have been thought to reflect an increasing proportion of glial cells in the aging brain (Robinson et al., 1971; Benedetti and Keane, 1980; Fowler et al., 1980; Irwin et al., 1992). As shown in Figure 65-2, increased glial MAO-B activity may lead to a higher rate of dopamine turnover and to an age-related decrease in dopaminergic signal transmission; these changes may predispose to parkinsonism, particularly if they eventually result in the damage of dopaminergic neurons. Thus, the involvement of astrocytes in dopamine metabolism not only may contribute to the loss of dopaminergic activity observed in Parkinson's disease, but it may also explain, at least in part, the role of aging as a risk factor for parkinsonism (Kurland *et al.*, 1973).

MAO-dependent dopamine metabolism may also represent a major source of oxidative stress by generating H_2O_2 (Figure 65-1). Experimental evidence supports this hypothesis. For example, enhanced dopamine turnover caused by reserpine or haloperidol has been shown to result in increased levels of oxidized glutathione (GSSG) in the mouse striatum (Spina and Cohen, 1989). H_2O_2 production via MAO is probably responsible for this effect since oxidative stress could be prevented by the MAO inhibitors clorgyline and deprenyl (Spina and Cohen 1989; Cohen and Spina 1989). This MAO-related pathway of oxidative damage has long been proposed to play an important role in neuronal cell death in Parkinson's disease (Cohen, 1983). The extraneuronal localization of MAO B (Levitt et al., 1982; Westlund et al., 1985) also suggests the involvement of glial cells in these processes: H_2O_2 could be generated by astrocytes and, because of its ability to cross cell membranes (Halliwell and Gutteridge, 1984), it might then attack its neuronal targets (Figures 65-2 and 65-3).

It is noteworthy that, while being a source for H_2O_2, glial cells may also exert a protective function against oxidative stress. This is because glutathione, a key molecule in the detoxification of H_2O_2, appears to be present in relatively high concentrations in astrocytes as compared to neuronal cells (Slivka et al., 1987; Raps et al., 1989). Reduced glutathione (GSH) is the source of electrons for the reduction of H_2O_2 catalyzed by glutathione peroxidase, and depletion of GSH levels has been associated with a greater vulnerability of cells to oxidative stress (Orrenius et al., 1985). It is potentially relevant, therefore, that decreased levels of glutathione have been reported in the brain and in the substantia nigra of parkinsonian patients as compared to control subjects (Perry et al., 1982; Riederer et al., 1989), leading to the speculation that Parkinson's disease may be a disorder due to nigral glutathione deficiency (Perry et al., 1982). This impairment of defense mechanisms against H_2O_2 accumulation may reflect a specific feature of glial cells in the parkinsonian brain. Furthermore, due to the possible involvement of astrocytes in both the generation and detoxification of H_2O_2, the presence of high GSH levels in these cells may protect them from oxidative injury; in contrast, lack of GSH in neuronal cells may predispose them to be damaged by the H_2O_2 formed in the process of dopamine turnover. This concept that H_2O_2 might cause different cytotoxic effects in astrocytes as compared to neurons due to the presence or lack, respectively, of glutathione is schematized in Figure 65-3.

The possibility that nigrostriatal dopaminergic neurons may be particularly vulnerable to oxygen radical production does not necessarily imply that

Aging

Glial proliferation

Increased MAO B activity

Metabolic activation of neurotoxicants

Increased rate of dopamine metabolism

Increased H_2O_2 production

Oxidative stress

Neuronal damage

Neuronal damage

Loss of dopaminergic activity

FIG. 65-2. Possible relationships between glial cells, monoamine oxidase, oxidative stress, and loss of dopaminergic activity in Parkinson's disease.

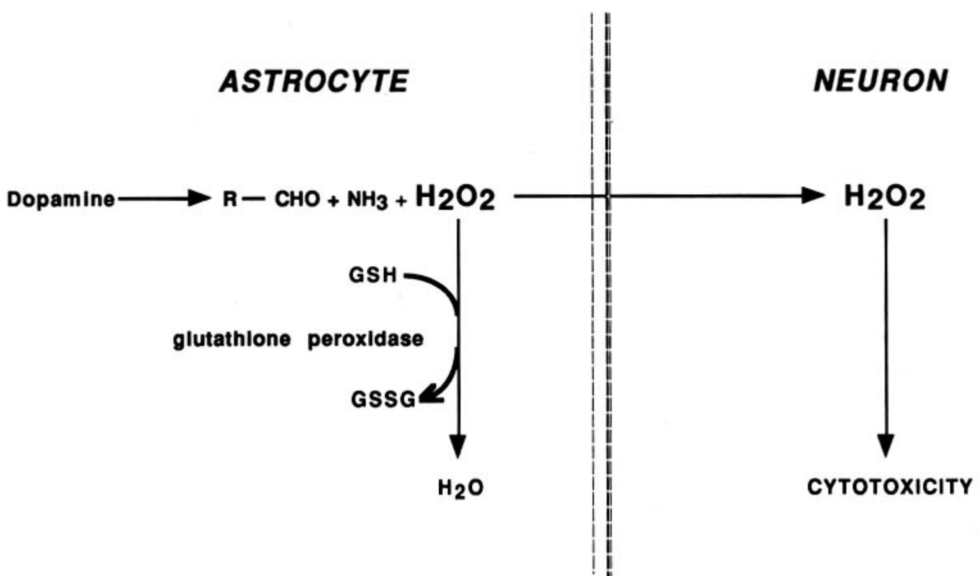

FIG. 65-3. Fate of the hydrogen peroxide (H_2O_2) generated in glial cells via monoamine H_2O_2 can be reduced to H_2O at the expense of glutathione (GSH) within astrocytes, or it may cross astrocyte and neuronal membranes. The lack of glutathione may make neurons more vulnerable to the cytotoxic effects of H_2O_2.

oxidative stress is the prominent cause of neurodegeneration in Parkinson's disease. Indeed, the free radical hypothesis for the pathogenesis of idiopathic parkinsonism remains quite controversial (Caine, 1992; Fahn and Cohen, 1992). More recently, toxic mechanisms other than oxidative stress have been suggested to play a role in nigrostriatal neurodegeneration. In particular, an impairment of mitochondrial energy production and/or the activation of excitatory amino acid receptors have been hypothesized. These novel pathogenetic hypotheses mostly stem from studies on MPTP neurotoxicity, and are discussed in greater detail in the next section.

MPTP-INDUCED PARKINSONISM

It is not surprising that the discovery of MPTP in 1982 immediately prompted great expectations among scientists working in the field of neurodegenerative disorders. Individuals who injected themselves with batches of "synthetic heroin" contaminated with MPTP developed a clinical syndrome virtually indistinguishable from severe idiopathic parkinsonism (Langston et al., 1983). It was therefore predicted that MPTP poisoning would become an excellent model for elucidating the process of neurodegeneration in human brain. The clinical similarities between MPTP-induced and idiopathic parkinsonism have since been further characterized (Tetrud et al., 1989). These similarities not only concern the major features of the disorder, but include more subtler signs, such as hypomimia, seborrhea, and micrographia. Moreover, MPTP patients responded to levodopa therapy and developed the typical side effects of this treatment, including end-of-dose "wearing off" and peak-dose dyskinesia. Positron emission tomography (PET) scanning has also revealed a reduction in striatal radioactivity in individuals exposed to MPTP as compared to age-matched controls (Caine et al., 1985).

The ability of MPTP to damage the nigrostriatal dopaminergic system as a primary target has been confirmed by both neurochemical measurements and neuropathologic studies in the monkey and rodent models. A severe and long-lasting depletion of striatal dopamine is consistently observed after systemic administration of MPTP to monkeys (Burns et al., 1983; Jenner et al., 1986; Irwin et al., 1990) or mice (Heikkila et al., 1984a; Melamed et al., 1985; Irwin et al., 1992). Whether or not MPTP is able to cause a regional pattern of dopamine loss in monkeys similar to that observed in Parkinson's disease remains controversial (Pifl et al., 1988), although a more pronounced dopamine depletion in the putamen than the caudate nucleus has been reported by Irwin et al., (1990).

From the pathologic point of view, the MPTP monkey model has revealed striking similarities as well as some differences with Parkinson's disease. The main similarities are (1) a marked destruction of neurons in the zona compacta of the substantia nigra (Burns et al., 1983; Langston et al., 1984a); (2) the occurrence of nerve cell loss in the locus ceruleus in the majority of MPTP-treated squirrel monkeys (Forno et al., 1986; Forno et al., 1993); (3) the presence of glial scars in the areas most severely affected by neurodegeneration (Forno et al., 1992), and (4) a more extensive loss of [^{3}H]mazindol binding in the putamen as compared to the caudate nucleus (Moratalla et al., 1992). The most relevant neuropathologic difference is the absence of typical Lewy bodies in animals exposed to MPTP. Abnormal nerve cells with inclusion bodies have been described in MPTP-treated squirrel monkeys (Forno et al., 1986; Forno et al., 1993); these inclusions are not a consistent finding, however, and do not display the morphological and immunocytochemical features of Lewy bodies.

Mechanisms of MPTP Neurotoxicity

Shortly after its discovery, MPTP was found to be converted by MAO-B into its fully oxidized 1-methyl-4-phenylpyridinium (MPP^+) metabolite (Chiba et al., 1984). MPP^+ represents the ultimate mediator of MPTP neurotoxicity, since (1) the damaging effects of MPTP exposure can be prevented by MAO-B inhibitors (Heikkila et al., 1984b; Langston et al., 1984c; Cohen et al., 1985), and (2) MPP^+ itself induces toxic effects similar to those seen after MPTP treatment (Langston et al., 1984b; Markey et al., 1984; Bradbury et al., 1986; Wu et al., 1992). The critical role played by MPP^+ in MPTP toxicity may also explain, at least in part, the selective action of MPTP toward the nigrostriatal dopaminergic system: MPP^+ has been shown to be actively taken up by dopaminergic neurons (Javitch et al., 1985) and it could be selectively accumulated within neurons of the substantia nigra also due to its binding to neuromelanin (D'Amato et al., 1986).

The biochemical mechanisms of cell death caused by MPP^+ have been the subject of some controversy. It was initially thought that MPP^+-induced cytotoxicity may result from free radical generation and consequent oxidative stress (Kopin et al., 1986). This hypothesis was suggested by the similarity of the chemical structure of MPP^+ with that of the herbicide paraquat, a known oxygen radical-generating

compound (Di Monte and Smith, 1988). Also, the possible involvement of oxidative stress in MPTP/MPP^+ toxicity appeared particularly attractive due to the suggested role of oxygen radicals in the pathogenesis of idiopathic parkinsonism as well. Subsequent studies have revealed significant differences in the mechanisms of cytotoxicity of MPP^+ and paraquat (Di Monte and Smith, 1988), however, and convincing evidence for a critical contribution of oxidative stress to MPTP/MPP^+ toxicity is still lacking.

It is now widely accepted that MPP^+-induced cell death may be a consequence of its ability to impair energy production by mitochondria. MPP^+ can be accumulated into the mitochondrial matrix, probably via the mitochondrial transmembrane potential (Ramsay and Singer, 1986; Sayre, 1989), and then it is capable of blocking the electron flow of the respiratory chain at the level of complex I (Nicklas et al., 1985). It is possible that an increased rate of oxygen radical generation may result from the interaction of MPP^+ with mitochondrial respiration and may contribute to the damaging effects of MPP^+ within these organelles (Cleeter et al., 1992). However, it is also quite evident that the major consequence of the action of MPP^+ as a mitochondrial poison is a failure of the cell to generate sufficient energy to support its function. Indeed, MPP^+ cytotoxicity has been directly linked to ATP depletion in a variety of *in vitro* systems (Di Monte et al., 1986; Denton and Howard, 1987; Wu et al., 1992), and more recently, MPP^+-induced ATP loss has been demonstrated to occur in the mouse brain after systemic administration of MPTP (Chan et al., 1993b).

The ability of MPP^+ to impair mitochondrial energy production may also help to elucidate recent findings suggesting a role of excitatory amino acid receptor activation in MPTP neurotoxicity (Turski et al., 1991; Zuddas et al., 1992). Adenosine triphosphate (ATP) depletion caused by MPP^+ could activate *N*-methyl-D-aspartate (NMDA) receptors by increasing the release and/or decreasing the reuptake of excitatory amino acids (Nicholls and Attwell, 1990). Furthermore, the membrane depolarization resulting from ATP depletion could relieve the voltage-dependent Mg^{2+} block of NMDA receptors, thus increasing their sensitivity to excitatory amino acids (Henneberry et al., 1989; Zeevalk et al., 1991). The role of excitatory amino acids in MPTP-induced neurodegeneration remains controversial (Sonsalla et al., 1989; Kupsch et al., 1992; Storey et al., 1992; Chan et al., 1993a) and certainly requires further clarification. However, it is noteworthy that the relationship between a failure of energy production and excitatory amino acid receptor activation has been hypothesized to underlie neuronal damage in human neurodegenerative disorders (Beal, 1992). Thus, studies with the MPTP model may once more contribute to enhance our understanding of the processes of cell degeneration in neurologic diseases.

Role of Glial Cells in MPTP Biodisposition

As mentioned earlier, MPTP is not toxic itself but needs to undergo a two-step process of metabolic activation in order to damage tissues. First, MPTP is converted to the 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) intermediate, and then $MPDP^+$ is further oxidized to the toxic metabolite MPP^+. The conversion of MPTP to $MPDP^+$ is catalyzed by MAO-B (Chiba et al., 1984), whereas no enzymatic activities and/or catalytic factors have been identified to date to be responsible for the subsequent formation of MPP^+. The role of MAO-B in the initial oxidation of MPTP to $MPDP^+$ points to astrocytes as a primary locus for MPTP metabolism. This reaction could also take place within MAO-B containing serotonergic neurons (Levitt et al., 1982; Westlund et al., 1985). The contribution of these neuronal cells to MPP^+ formation is unlikely to be significant, however, since studies in mice have indicated that lesions of serotonergic neurons do not attenuate MPTP neurotoxicity (Melamed et al., 1986; Brooks et al., 1989).

The ability of glial cells to bioactivate MPTP has been demonstrated by *in vitro* metabolic studies with primary astrocyte cultures. Astrocytes in culture have been shown to generate MPP^+ from MPTP (Ransom et al., 1987; Schinelli et al., 1988) via a MAO-dependent pathway (Ransom et al., 1987; Marini et al., 1989). Subsequent studies have revealed that, in addition to MPP^+, two other metabolites can be measured in astrocyte cultures exposed to MPTP, namely, $MPDP^+$ and MPTP *N*-oxide (Di Monte et al., 1991). Production of $MPDP^+$ is blocked by MAO inhibitors (Di Monte et al., 1991); since this inhibition prevented MPP^+ formation as well, data are consistent with the role of $MPDP^+$ as an intermediate metabolite in the bioactivation of MPTP. MPP^+ could also be generated after direct addition of $MPDP^+$ to astrocyte cultures (Wu et al., 1992); this reaction was unaffected by MAO inhibitors, however, indicating that MAO is involved only in the first step of MPTP oxidation to MPP^+. In fact, since the rate of oxidation of $MPDP^+$ to MPP^+ was found to be similar in the presence or absence of cells, a nonenzymatic mechanism of conversion appears to be quite likely (Wu et al., 1992).

The ability of astrocytes *in vitro* to generate MPTP *N*-oxide has been further characterized by studies

showing that this metabolite is probably the product of MPTP conversion via the flavin-containing monooxygenase (Di Monte et al., 1991). The role of this metabolic pathway in MPTP metabolism *in vivo* remains to be elucidated. It is noteworthy, however, that, at least *in vitro*, flavin-containing monooxygenase activity can oxidize MPTP without generating the toxic MPP^+ metabolite; this suggests that metabolic processes within astrocytes may be involved in both bioactivation and detoxification of MPTP.

If glial cells play an essential role in the conversion of MPTP to MPP^+, the mechanism by which this toxic metabolite reaches the extracellular space and thus becomes available for accumulation into dopaminergic neurons needs to be elucidated. Any proposed mechanism should take into consideration the fact that MPP^+ would not be expected to cross cell membranes easily due to its charged chemical structure. It is possible that leakage of MPP^+ into the extracellular compartment may be a consequence of damage to the very same glial cells which generate this toxic metabolite. This relatively simple explanation is supported by histopathological evidence *in vivo* showing acute damage of astrocytes after systemic administration of MPTP to mice (Adams et al., 1989). The ability of both MPTP and MPP^+ to damage glial membranes is further indicated by results with primary astrocyte cultures in which increasing levels of lactate dehydrogenase (a cytosolic enzyme) were measured extracellularly after treatment with either of the two compounds (Di Monte et al., 1992b; Wu et al., 1992).

In vitro studies have revealed that, in the presence of MPTP, significant levels of MPP^+ can be accumulated in the extracellular space well before any apparent damage to astrocyte membranes (Di Monte et al., 1991; Di Monte et al., 1992b). Thus, mechanism(s) other than cytotoxicity are likely to contribute to the biodisposition of MPP^+. Abundant evidence now suggests that MPP^+ itself can cross cell membranes. Indeed, recent studies have revealed that (1) the positive charge of MPP^+ is delocalized throughout the pyridinium ring, resulting in a certain degree of lipid and membrane solubility (Reinhard et al., 1990), (2) MPP^+ can be measured intracellularly after its addition to astrocyte cultures (Wu et al., 1992), and (3) when astrocytes are pretreated with MPTP and then washed and incubated in MPTP-free fresh medium, intracellular levels of MPP^+ rapidly decline while extracellular MPP^+ concentrations rise in a stoichiometric manner (Di Monte et al., 1992c).

A third mechanism that could account for the presence of MPP^+ in the extracellular compartment may involve the ability of $MPDP^+$ to cross cell membranes (Di Monte et al., 1992c). $MPDP^+$ could be generated intracellularly from MPTP by MAO and it could cross astrocyte membranes, possibly in the form of the lipophilic free base 1,2-MPDP. Then, MPP^+ may be formed directly outside the cells via autoxidation of $MPDP^+$ (Figure 65-4). A rapid extracellular conversion of the unstable $MPDP^+$ metabolite is suggested by findings showing that $MPDP^+$ levels in the medium remain relatively low and constant throughout the incubation of astrocyte cultures in the presence of MPTP (Di Monte et al., 1991).

Taken together, experimental data are compatible with a primary role of glial cells in MPTP biodisposition, having characterized the process of metabolic activation of MPTP to MPP^+ as well as the mechanisms of MPP^+ "delivery" into the extracellular compartment. It is noteworthy that MPP^+ may become available for neuronal uptake without damaging necessarily glial cells. This possibility may explain why astrocytes are not a more obvious target for MPTP toxicity *in vivo* (Forno et al., 1992). It also underlines the role of mechanisms for "trapping" MPP^+ within neurons (*e.g.*, the continuous reuptake of MPP^+ and/or its binding to neuromelanin) in the selective action of MPTP (Figure 65-4).

Role of Glial Cells in MPTP-Induced Neurotoxicity

Once MPP^+ is accumulated into dopaminergic neurons, the subsequent series of biochemical/toxic events is likely to involve inhibition of mitochondrial respiration and consequent failure of energy supplies. Could glial cells play a role also in this final stage of MPTP neurotoxicity? The answer to this question necessarily involves speculative mechanisms, since our knowledge of MPP^+ action *in vivo* remains quite scarce and the role of astrocytes in brain energy metabolism has just started to be explored in detail. Glial cells have long been proposed to act as an energy source for neurons, however (Kuffler et al., 1984). One mechanism that might account for this function is their ability to accumulate energy substrates in the form of glycogen (Guth and Watson, 1968; Cataldo and Broadwell, 1986); in conditions of increased neuronal activity, glycogenolysis may be activated in astrocytes in order to sustain energy demand (Magistretti et al., 1986). Thus, it can be hypothesized that glial cells may provide metabolic support to counteract MPTP-induced failure of energy supplies. This metabolic support may only be partial and/or temporary, however, and therefore it may not prevent irreversible damage of neurons exposed to high levels of MPP^+ for a longer period of time.

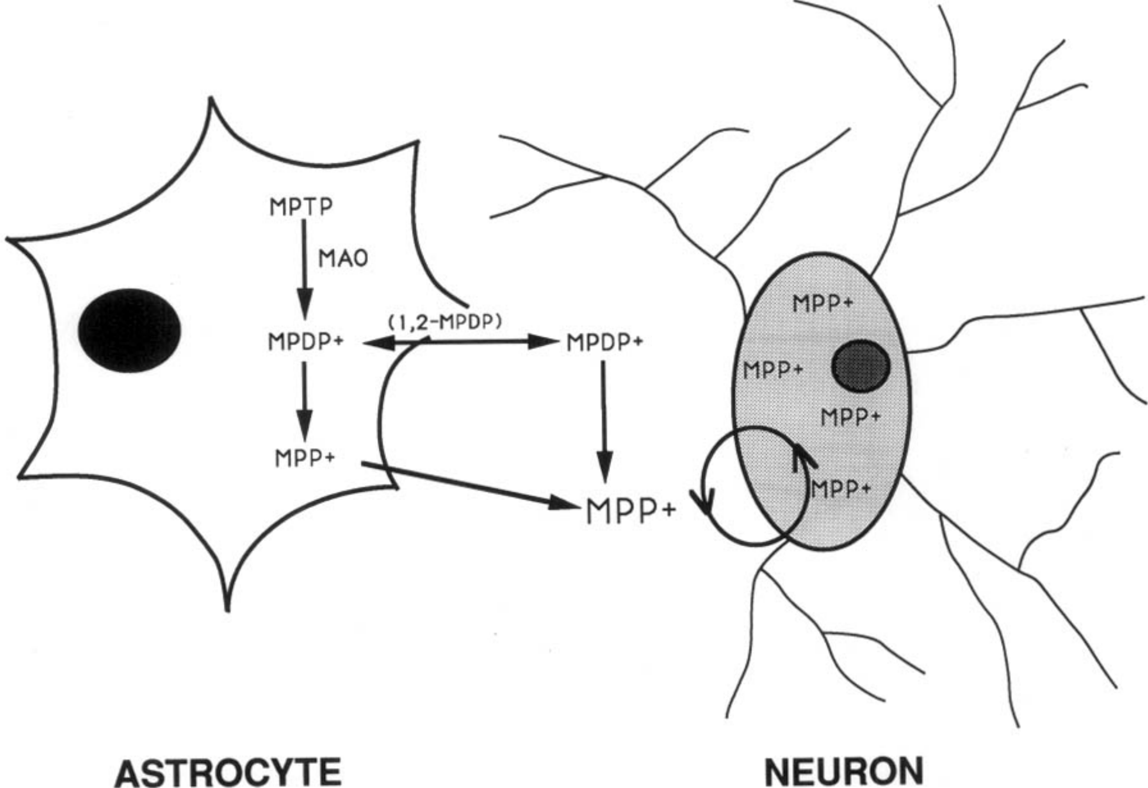

FIG. 65-4. Generation and disposition of MPP^+ as a consequence of MPTP metabolism in astrocytes. The ability of dopaminergic neurons to take up and retain MPP^+ is likely to play a role in their selective susceptibility to MPTP/MPP^+ neurotoxicity.

As a consequence of impaired energy production, MPTP/MPP^+ action may involve excitatory amino acid receptor activation, suggesting another possible role for glial cells in MPTP-induced neurotoxicity. Glial cells are able to accumulate glutamate via a high-affinity uptake system (Hertz, 1979) and to convert glutamate to glutamine by glutamine synthetase (Martinez-Hernandez et al., 1977). Therefore, if neuronal damage is related to an increased release of excitatory amino acids and/or an increased sensitivity of NMDA receptors to excitatory amino acids, expression of MPP^+ neurotoxicity may depend on the ability of astrocytes to modify the extracellular concentrations of glutamate. Interestingly, this function of glial cells may conceivably result either in a protective or a damaging role. Astrocytes may protect against MPP^+-induced excitotoxic injury by scavenging glutamate from the extracellular space, thereby reducing the exposure of NMDA receptors to this excitatory amino acid. However, active uptake of glutamate by glial cells requires ATP and therefore may become less efficient after MPP^+ exposure; such a mechanism would contribute to increase the extracellular levels of excitatory amino acids and could possibly enhance excitotoxicity. Another mechanism by which astrocytes may exacerbate excitotoxic neuronal damage caused by MPTP/MPP^+ is suggested by the finding that injured glial cells may themselves release excitatory amino acids such as glutamate, aspartate, and taurine (Kimelberg et al., 1990). Studies in the past few years have already focused on the role of excitatory amino acids and glial cells in the hypoxia/ischemia model of neuronal injury (Choi, 1988; Yu et al., 1992); information acquired from these studies may provide directions for future work on the possible involvement of astrocytes and excitatory amino acids in MPTP-induced "chemical hypoxia."

CONCLUSIONS

The role of glial cells in idiopathic and MPTP-induced parkinsonism has been discussed separately in the previous sections of this review. It is important to underline, however, that, besides the clinical and neuropathologic similarities, idiopathic and MPTP-induced parkinsonism share a number of relevant features. Furthermore, the MPTP model has provided valuable clues on mechanisms of neuronal injury that have just started to be investigated in the idiopathic disorder. Some of these common features

and mechanisms point to astrocytes as possible active participants in the process of neurodegeneration and therefore will be summarized next.

MAO Activity

The involvement of MAO in the pathogenesis of neurodegeneration appears to be a recurrent theme in both idiopathic and MPTP-induced parkinsonism. This is because of (1) the important function of MAO in dopamine metabolism, (2) the generation of H_2O_2 as a product of MAO catalytic activity, and (3) the ability of MAO to bioactivate neurotoxicants such as MPTP. For all these reasons, in 1986 and 1987, two separate clinical trials were initiated in order to test the effects of chronic administration of the MAO-B inhibitor deprenyl on the course of Parkinson's disease. Results of both these studies have indicated that deprenyl treatment delays the need for levodopa therapy in patients with early Parkinson's disease (Tetrud and Langston, 1989; Parkinson Study Group, 1989), adding strong support to the view that MAO activity may be a "risk factor" for the degeneration of dopaminergic neurons.

The function of glial cells in both MAO-dependent dopamine metabolism and MPTP biotransformation probably represents the strongest evidence in favor of their involvement in the pathogenesis of human parkinsonism. A number of questions remain to be explored, however, on the activity of glial MAO in relation to dopaminergic neurodegeneration. For example, regional differences in glial MAO-B expression need to be investigated as a possible reason for selective neuronal damage. Also, the function of astrocytes as a source of H_2O_2 as well as in defense against H_2O_2 accumulation should be further characterized. Finally, the possibility that metabolic pathway(s) other than the MAO-dependent one may contribute to the conversion of MPTP to MPP^+ in glial cells requires attention. This possibility is suggested by studies both *in vivo* and *in vitro* showing that MPP^+ generation cannot be completely blocked by MAO inhibitors (Langston et al., 1984c; Markey et al., 1984; Di Monte et al., 1991).

Aging

Another important feature shared by idiopathic parkinsonism and MPTP neurotoxicity is an increased susceptibility with age. Aging has long been acknowledged as the only unequivocal risk factor for Parkinson's disease (Kurland et al., 1973) and, interestingly, the sensitivity to MPTP-induced dopamine depletion and damage to dopaminergic neurons has been shown to be enhanced in older animals (Jarvis and Wagner, 1985; Gupta et al., 1986; Irwin et al., 1992). These age-related effects of MPTP have been correlated with changes in brain levels of MAO-B (Irwin et al., 1992). Since an increased expression of MAO-B activity has also been measured in the aging human brain (Robinson et al., 1971; Fowler et al., 1980), it is conceivable that MAO-B changes may play a prominent role in making the aged brain more vulnerable to nigrostriatal degeneration. Other mechanisms might contribute to this effect, however. For example, recent work in our laboratory has revealed that mitochondrial energy production declines with age in the monkey striatum (Di Monte et al., 1993). These results are particularly intriguing given the likely involvement of mitochondrial poisoning in MPTP action and the possible occurrence of mitochondrial complex I deficiency in Parkinson's disease as well (see next section on mechanisms of neurodegeneration).

As noted earlier, the age-related increase in MAO-B activity has been attributed to a greater proportion of MAO-B-containing astrocytes in the older brain (Benedetti and Keane, 1980; Fowler et al., 1980; Irwin et al., 1992), suggesting that aging processes related to glial cells may ultimately contribute to the pathogenesis of parkinsonism. This possibility reveals another area for relevant future investigations. Do age-dependent changes in astrocyte functions and characteristics incrase the risk for neuronal damage? Such changes might include a decline in energy production which, in turn, may affect the ability of astrocytes to sustain neuronal energy demand in conditions of "metabolic stress."

Mechanisms of Neurodegeneration

The final events leading to irreversible neuronal damage in human parkinsonism are quite controversial and remain essentially unknown. The oxidative stress hypothesis, while based on a number of valid considerations and indirect evidence, is apparently inconsistent with our knowledge about the mechanism of action of MPTP. In the MPTP model, the ability of MPP^+ to inhibit mitochondrial respiration is more likely to be the primary event leading to neuronal damage, and findings of recent studies in humans have raised the possibility that a similar mechanism may play a role in Parkinson's disease as well. Indeed, a decrease in mitochondrial respiratory chain enzyme activities has been measured in different tissues of parkinsonian as compared to control subjects (Bindoff et al., 1989; Parker et al., 1989; Schapira et al., 1990). In the brain, this decrease ap-

pears to be selective both for the substantia nigra and for mitochondrial complex I (Schapira et al., 1990).

The controversy over the mechanism of neurodegeneration in human parkinsonism may only be apparent, however, and could certainly be reconciled. There is little doubt that links exist between oxygen radical production and mitochondrial damage: mitochondria are primary targets for oxidative stress and, vice versa, oxygen radicals may be generated as a consequence of impaired mitochondrial activity (Di Monte et al., 1992a). Furthermore, as already mentioned, other damaging events, such as the activation of excitatory amino acid receptors, may be triggered by mitochondrial damage and/or oxidative stress. Thus, different toxic mechanisms may be set in motion by the initial biochemical change and may all contribute to neurodegeneration. Even more importantly, the process of neuronal damage may ultimately be affected by agents acting against oxidative stress as well as by compounds able to supplement cellular energy supplies or attenuate excitotoxicity.

It is noteworthy that glial cells may play a role in all of these toxic mechanisms. They could contribute to H_2O_2 production (via the MAO-dependent dopamine and MPTP metabolism) while representing a primary locus for H_2O_2 scavenging by glutathione peroxidase; they may provide energy support for neurons in conditions of metabolic stress, and protect against NMDA receptor activation by the active uptake of glutamate. Additional studies on astrocyte function in these processes may not only contribute to our understanding of the pathogenesis of parkinsonism, but it even seems possible that such studies will help us to unravel the biochemical and toxic basis of other neurodegenerative diseases as well.

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66 Inherited disorders of myelination of the central nervous system

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The elaboration of the myelin sheath by the oligodendrocyte and Schwann cell in the central and peripheral nervous systems is one of the most remarkable and fascinating aspects of neural development. In the central nervous system the oligodendrocyte, which has a small cell soma, supports as many as 50 flattened expansions of spirally wrapped, tightly compacted myelin membrane via slender cytoplasmic "arms" (Morell and Norton, 1980; Wood and Bunge, 1984). In marked contrast, myelination in the peripheral nervous system is accomplished by Schwann cells, which generate only one myelin segment. During myelination, oligodendrocyte and Schwann cells produce vast quantities of plasma membrane and insert into the lipid matrix the myelin-specific polypeptides that participate in the initial organization and subsequent maintenance of the myelin sheath. In order to initiate the myelination proce^s, myelinating cells must induce and express the genes encoding the myelin proteins at high levels, and must orchestrate the insertion of these polypeptides into the rapidly expanding regions of the plasma membrane that will comprise the "myelin domains" of the cell surface. The critical requirement for myelination of the nervous system to proceed according to schedule and to be maintained is demonstrated in inherited and acquired diseases where myelin does not develop normally or is lost as a result of demyelinating process. In both instances, the lack of insulation of axons leads to disruption of neurological function with debilitating and often fatal consequences.

Our understanding of myelination and the myelin sheath has been greatly advanced in the last decade, following the application of molecular biological techniques to study this developmental process. In particular, the cloning of the major myelin genes of the peripheral nervous system and central nervous system have provided the tools with which to begin to explore the patterns of expression of the myelin genes and their developmental regulation (Sutcliffe, 1987; Campagnoni and Macklin, 1988; Lemke, 1988).

While much information on myelination has been derived from the use of such molecular techniques in the study of the normal nervous system, mutant animals in which this process is disrupted have provided alternative approaches to study myelination (Baumann, 1980; Duncan et al., 1990). These animals, collectively known as the myelin mutants, have been crucial to understanding the function of individual myelin proteins, both in the structure of the myelin sheath and in the myelination process, particularly in the central nervous system. However, the correlation between the genotype and resultant phenotype of the myelin mutants is often not straightforward; in the X-linked mutants, for instance, the relationship between described mutations and abnormal oligodendrocyte development and early oligodendrocyte death are not yet explained (Duncan, 1990; Hudson and Nadon, 1992; Skoff and Knapp, 1992). Findings such as these have provided many new opportunities to study the role(s) of individual myelin proteins in the division, maturation, and survival of the myelinating cell. It is likely that these issues will only be resolved by the combination of molecular and cellular techniques.

The myelin mutants can be divided according to their mode of inheritance into two major categories, the X-linked mutants and those that are inherited as autosomal recessive traits. The X-linked mutants form the largest and most homogeneous group as their genetic defects are linked to a single chromosome and in the majority a mutation in the proteolipid protein gene has been identified (Hudson and Nadon, 1992). In contrast to the great advances made in recent studies of the X-linked mutants, less is known about the molecular defects in those of autosomal origin. This chapter presents the X-linked mutants as a group and describes the known features of the autosomal recessive myelin mutants, particularly the effects of these mutations on neuroglia. The molecular genetics of these mutations are discussed in Chapters 38 and 39.

THE X-LINKED MYELIN MUTANTS

The X-linked myelin mutants have been discovered in a wide range of species, including man (Pelizaeus-

Merzbacher disease) (Hudson and Nadon, 1992), dog (shaking pup [*sh pup*]) (Griffiths et al., 1981), pig (type A III hypomyelinogenesis congenita) (Blakemore et al., 1974), rat (myelin-deficient [*md*]) (Dentinger et al. 1982; Jackson and Duncan, 1988), rabbit (paralytic tremor [*pt*]) (Taraszewska, 1988), mouse (jimpy [*jp*]) and its alleles, myelin synthesis deficiency (jp^{msd}) (Skoff and Knapp, 1992) and rumpshaker (jp^{rsh}) (Griffiths et al., 1990). In addition to this list, a new proteolipid protein (PLP) mouse mutant called jp^{4J} has very recently been reported (Billings-Gagliardi et al., 1994). This new mutant has a shorter life-span than *jp* and appears to have a more marked deficiency of myelin and oligodendrocytes.

The severity of the myelin defects leads to the majority of these mutations being lethal, but the *sh pup*, *pt* rabbit, and jp^{rsh} mouse are able to live for extended periods of time and are fertile. In general, the longer life span of these mutants relates to the greater amount of myelin in the central nervous system. Thus, the *md* rat and *jp* mouse, which have the least myelin die earlier than jp^{rsh} and the *sh pup*, which have considerably more. While the correlation between lack of myelin and the early demise of the affected mutant holds true overall, a longer-lived "strain" of the *md* rat has arisen, which lives for up to three times as long as the original "strain" of *md* rat, although it has no more myelin (Duncan et al., 1995).

The major phenotypic abnormalities seen in the X-linked mutants are in (1) the amount of myelin, (2) myelin structure, (3) oligodendrocyte number and cytoplasmic abnormalities, and (4) astrocytic responses. Each of these is presented in the following sections and differences between mutants discussed. A summary of these changes is presented in Table 66-1.

Amount of Myelin

There are great variations in the severity of the myelin defects seen in the central nervous system of the X-linked mutants, ranging from little to no myelin in the *md* rat, to considerable amounts of myelin in jp^{rsh}. Figure 66-1 demonstrates this range of myelin deficiencies. In the most severely affected mutants, the later myelinating parts of the central nervous system, such as the brain and optic nerves have consistently less myelin than the spinal cord. This is not surprising, as both the *md* rat and *jp* mouse die at 23 to 25 days of age, at a time when myelination, in the normal animal, of more rostral areas such as the corpus callosum would still be occurring. In the most severely affected mutants, such as *jp* and the *md* rat, dysmyelination is the predominant abnormality with the majority of axons being unensheathed and those that are myelinated, having a poorly compacted myelin sheath (Figure 66-2). In

TABLE 66-1. *Summary of the Phenotypic Abnormalities in the X-Linked Myelin Mutants*

Phenotype	jp	jp^{msd}	jp^{rsh}	md rat	shaking pup	PMD
Amount of myelin	+	+/++	++++	+	+++	+/++?
Myelin structure	Dysmyelination compacted/ decompacted IPL	Same as jp	Hypomyelination pos/neg IPL	Dysmyelination neg IPL	Hypomyelination pos IPL	Dysmyelination ?IPL
Myelin immunolabeling	PLP-neg MBP-pos	PLP-neg MBP-pos	PLP-neg, DM20-pos MBP-pos	PLP-neg MBP-pos	PLP-pos MBP-pos	PLP-neg MBP-pos
Oligo number	↓↓ (but many immature oligos)	↓	Normal to increased	↓↓	↓	↓
Oligo structure	↑ Membranous profiles, etc.	? Distended RER	NAD	Distended RER	Distended RER	?Distended RER
Oligo proliferation	↑↑	NE	↑	↑	?↑	NE
Cell death	↑↑	↑	NAD	↑	?↑	ND
Gliosis	+++	?	NAD	++	++	++/+++
Microglia	↑↑↑	↑↑	NAD	↑↑	↑	?
Heterozygotes	Mosaic	NE	NAD	Mosaic	Mosaic	?

PMD, Pelizaeus-Merzbacher disease; PLP, proteolipid protein; MBP, myelin basic protein; RER, rough endoplasmic reticulum; Oligo, oligodendrocyte; IPL, intraperiod line; NAD, no abnormality detected; NE, not examined; +, o/occasional myelinated fibers; +++++, normal, neg. negative; pos, positive.

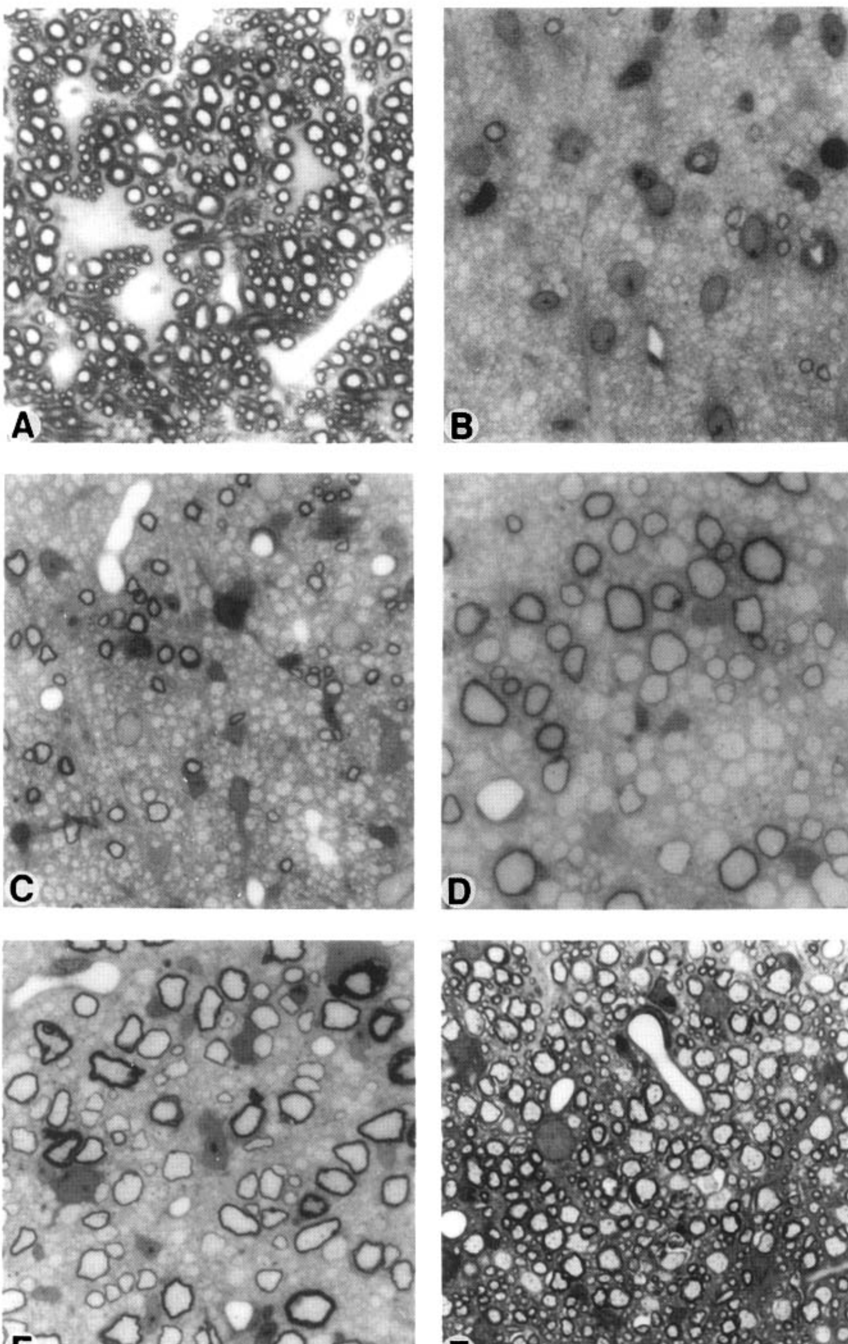


FIG. 66-1. Montage of similar areas from the ventral spinal cord white matter to show the range of myelin paucity seen in the X-linked mutants; (A) normal rat, (B) myelin-deficient rat, (C) jimpy mouse, (D) shaking pup, (E) type A III porcine hypomyelinogenesis congenita, (F) rumpshaker mouse. Mouse and rat sections from animals aged 16 to 20 days of age; pig and shaking pup ~8 weeks of age. Toluidine blue. All ×312. [Modified from Duncan (1990), with permission.]

the less severely affected mutants such as jp^{rsh}, hypomyelination is the predominant abnormality with most axons being myelinated but with inappropriately thin myelin sheaths (Figure 66-1).

Microscopic examination of the spinal cord of *jp* and the *md* rat shows that myelinated fibers are mainly seen in the ventral and lateral columns, often in a clumped pattern or less commonly as scattered fibers. In *jp* it has been estimated that the amount of myelin in the spinal cord is 1.6% of controls (Duncan and Hammang, 1989) but jp^{msd} has approximately twice the amount of myelin as *jp* (Billings-Gagliardi and Adcock, 1980). In the *sh pup*, qualitative comparisons suggest that more myelinated fibers are seen than in *jp* and the *md* rat, but as in the murine mutants, there can be marked animal-to-animal variation. Shaking pups have lived up to 23 months of age, and, while more central nervous system myelin may be seen in the older animals, there is persistent hypomyelination and lack

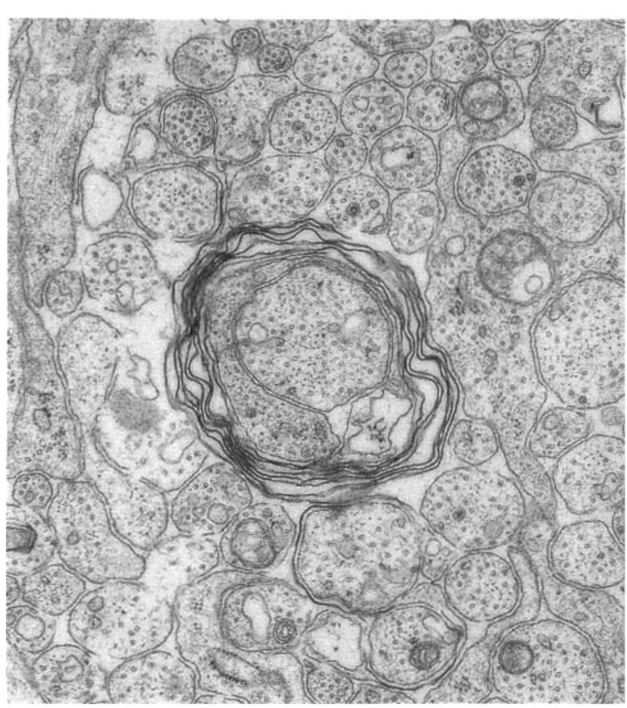


FIG. 66-2. Electron micrograph of a typical poorly compacted myelin sheath surrounded by many unensheathed axons from an *md* rat. These abnormalities are the hallmarks of dysmyelination. ×27,600. [From Duncan (1990), with permission.]

of ensheathement of many axons. The relationship between myelin sheath thickness and axonal diameter is often abnormal in the *sh pup*, with smaller axons frequently having thicker myelin sheaths than larger axons (Duncan and Griffiths, 1983). However, these smaller axons may also have shorter internodes, suggesting that mutant oligodendrocytes are restricted in the amount of membrane they can produce.

In jp^{rsh} there is much more myelin than in the other X-linked murine mutants (Griffiths et al., 1990). However, quantitative studies of myelin in the spinal cord and optic nerve have shown differences in development of the defect in these areas (Fanarraga et al., 1992). In the spinal cord, there is a progressive increase in the number of axons that are myelinated, and by 90 days most have a myelin sheath, although the larger diameter axons remain hypomyelinated. In the optic nerve, myelination commences normally and sheaths are appropriately thick, but many axons, even in 1-year-old mice, remain nonmyelinated. The reasons for these differences are not known, but it may relate to the fact that there are fewer oligodendrocytes in the optic nerve than normal in jp^{rsh}, whereas the converse is true in the spinal cord (Fanarraga et al., 1992). It may also relate to the apparent differences in the normal central nervous system between the numbers of axons myelinated by oligodendrocytes in the spinal cord compared to the brain (Remahl and Hildebrand, 1990) or to the presence of larger axons in the spinal cord. This difference between the myelin deficit in jp^{rsh} spinal cord and optic nerve remains to be resolved.

The *pt* rabbit has similar amounts of myelin to jp^{rsh}, with the main abnormalities in this mutant being a delay in myelination (Taraszewska, 1988). There is persistent hypomyelination and many small axons remain nonmyelinated. In Pelizaeus-Merzbacher disease in man, a detailed, high-resolution analysis of the amount of myelin has been impossible because of the lack of tissue for plastic embedding. In addition, there are numerous variants of Pelzaeus-Merzbacher disease with different genetic backgrounds (Hudson and Nadon, 1992), which complicates the picture. In certain forms of the disease, no myelin is seen grossly or in paraffin-embedded sections, but in others, the presumptive white-matter tracts have a patchy or "tigroid" appearance. Immunocytochemical labeling with antibodies to myelin proteins suggests that such patches are islands of myelin (Koeppen et al., 1987).

Myelin Structure and Composition

Just as with myelin volume, there is a marked variation in the structure of the myelin sheaths seen in the X-linked mutants. In the dysmyelinating mutants, there is only rare evidence of compact myelin, with most ensheathed axons being surrounded by uncompacted whorls of membrane (Figure 66-2). In both *jp* and its allele, jp^{msd} and the *md* rat, however, there are occasional fibers with well-compacted myelin sheaths, which have up to 20 myelin lamellae (Figure 66-3). On detailed examination of these sheaths, however, the intraperiod line is abnormally compacted and it is difficult to distinguish the major dense and putative intraperiod lines (Duncan et al., 1987b; Duncan and Hammang, 1989; Hudson and Nadon, 1992). However, in *jp*, jp^{msd}, and *md*, occasional stretches of the intraperiod line appear to have a normal structure. In *jp*, it has been suggested that well-compacted sheaths are usually seen in younger mice; in older *jp* mice, most myelin is poorly compacted (Nagara and Suzuki, 1982), which suggests that the sheaths are unstable.

In the mutants where hypomyelination is the predominant abnormality, myelin compaction is usually more normal, although many fibers have some loosening of compaction (Figure 66-2). In the *sh pup*, there is no evidence of an abnormality in the intra-

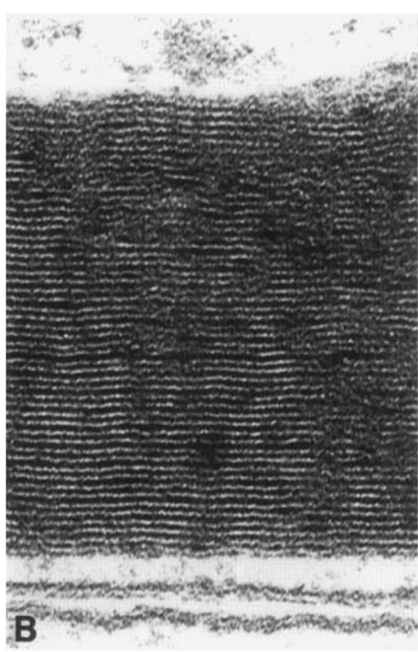


FIG. 66-3. Electron micrographs showing myelin sheaths from an (A) normal rat and (B) myelin-deficient rat spinal cord at 23 days of age. In the normal rat, a double intraperiod line is present, whereas in the *md* rat myelin there is a complete compaction of the intraperiod line, which cannot be distinguished from the major dense line. ×250,000. [Modified from Duncan et al. (1987b), with permission.]

period line, but in jp^{rsh} some sheaths show an abnormal compaction, whereas others do not (Griffiths et al., 1990).

Nodes of Ranvier in these mutants vary in their degrees of normality. In the *sh pup*, many heminodes are seen and the paranodal loops showed a number of abnormalities (Griffiths and Duncan, 1981). Frequent astrocytic interdigitations are seen at heminodes and this will be discussed later. Abnormalities in the paranodal axoglial junction have also been described in the *md* rat (Rosenbluth, 1987) as well as aberrant astrocytic invasion.

The protein composition of the myelin found in the X-linked mutants has been extensively investigated, particularly with regard to the presence or absence of proteolipid protein (PLP), as a severe reduction in PLP could relate directly to the lack of myelin. Biochemical data have shown that PLP is almost absent in the severely affected mutants (*jp* and the *md* rat) and that other myelin proteins, including myelin basic protein (MBP), are dramatically reduced (Quarles, 1990). Immunolabeling of myelin in *jp* and *md* with antibodies to PLP and MBP shows that the myelin present is PLP-negative but MBP-positive (Duncan et al., 1987b; Duncan and Hammang, 1989). The lack of PLP has been suggested to result in the abnormal compaction of the intraperiod line (Figure 66-3). Those mutants with more myelin present a more complex picture however. In the *sh pup*, myelin is PLP- and MBP-positive, but there appears to be less PLP than in wild-type myelin, (Yanagisawa et al. 1987) suggesting a delay or deficiency in incorporation of PLP into the myelin membrane. In jp^{rsh}, labeling with antibodies against only PLP or PLP/DM20 reveal different results. Staining of white matter in jp^{rsh} with an anti-PLP-specific antibody is markedly reduced compared with wild type (Griffiths et al., 1990). However, modest staining of myelin in jp^{rsh} is seen with the antibody recognizing PLP and DM20, which increases with age (Fanarraga et al., 1992). The key role that DM20 may play in jp^{rsh} and in the other X-linked mutants will be discussed further below.

In Pelizaeus-Merzbacher disease in man, immunolabeling of the scattered myelin present in the brain with antisera to MBP, myelin-associated protein (MAG), and cyclic nucleotidephosphodiesterase (CNP) is positive, but PLP-negative (Koeppen et al., 1987).

Oligodendrocytes - Lineage and Cytoplasmic Alterations

Much of the earlier work on *jp* and the other X-linked mutants centered around the description and estimates of the number of glia, in particular the oligodendrocytes. There is general agreement in the literature that there is a reduction in the number of mature oligodendrocytes in the X-linked mutants, with the exception of jp^{rsh} (Skoff and Knapp, 1992). However, there appears to be a significant population of cells, at least in *jp*, which label with galactocerebroside antibody, an early marker of the mature oligodendrocyte. Quantitative labeling of oligodendrocytes in the 15-day-old *jp* cerebellum la-

beled with a galactocerebroside antibody showed no statistical difference from controls in the number of positive cells. (Ghandour and Skoff, 1988). An increase in oligodendrocyte cell death between 15 and 25 days of age, the time at which *jp* usually dies, may account for the apparent paucity in oligodendrocytes as described by others. Ultrastructural studies of the *jp* central nervous system, and many of the other mutants have described many cells with features of immature oligodendrocytes (Figure 66-4), and there is a general inference that mutations in the PLP gene result in an abnormality of oligodendrocyte development (Nadon et al., 1990; Skoff and Knapp, 1992). In the *md* rat, there is also a reduction in the number of oligodendrocytes (Dentinger et al. 1985; Jackson and Duncan, 1988), and in the *sh pup*, quantitation of oligodendrocytes in the spinal cord also shows a reduction, although many of the oligodendrocytes appear mature (Duncan and Griffiths, 1983). The exception to this rule of oligodendrocyte reduction in the X-linked mutants is *jp^rsh^*, which has more oligodendrocytes than normal in the spinal cord, although slightly less in the optic nerve (Fanarraga et al., 1992).

While many oligodendrocytes appear immature in the X-linked mutants, others show cytoplasmic and nuclear changes, most of which are nonspecific signs of early cell death, but certain abnormalities are unique and could provide clues to the biochemical nature of the defect. The earlier literature, especially on *jp*, concentrated on the nonspecific ultrastructural changes in oligodendrocytes with cytoplasmic accumulation of lipids and membranous tubules (Skoff and Knapp, 1992). The *md* rat and *sh pup*, however, share a unique oligodendrocyte abnormality of rough endoplasmic reticulum (RER) (Dentinger et al. 1985; Duncan and Griffiths, 1983), which is distended and contains a floccular/proteinaceous material (Figure 66-5). The initial swelling of the RER is usually associated with accumulation of an electron dense substance in the cisterns (Figure 66-5A), but as the swellings enlarge, the stored material becomes

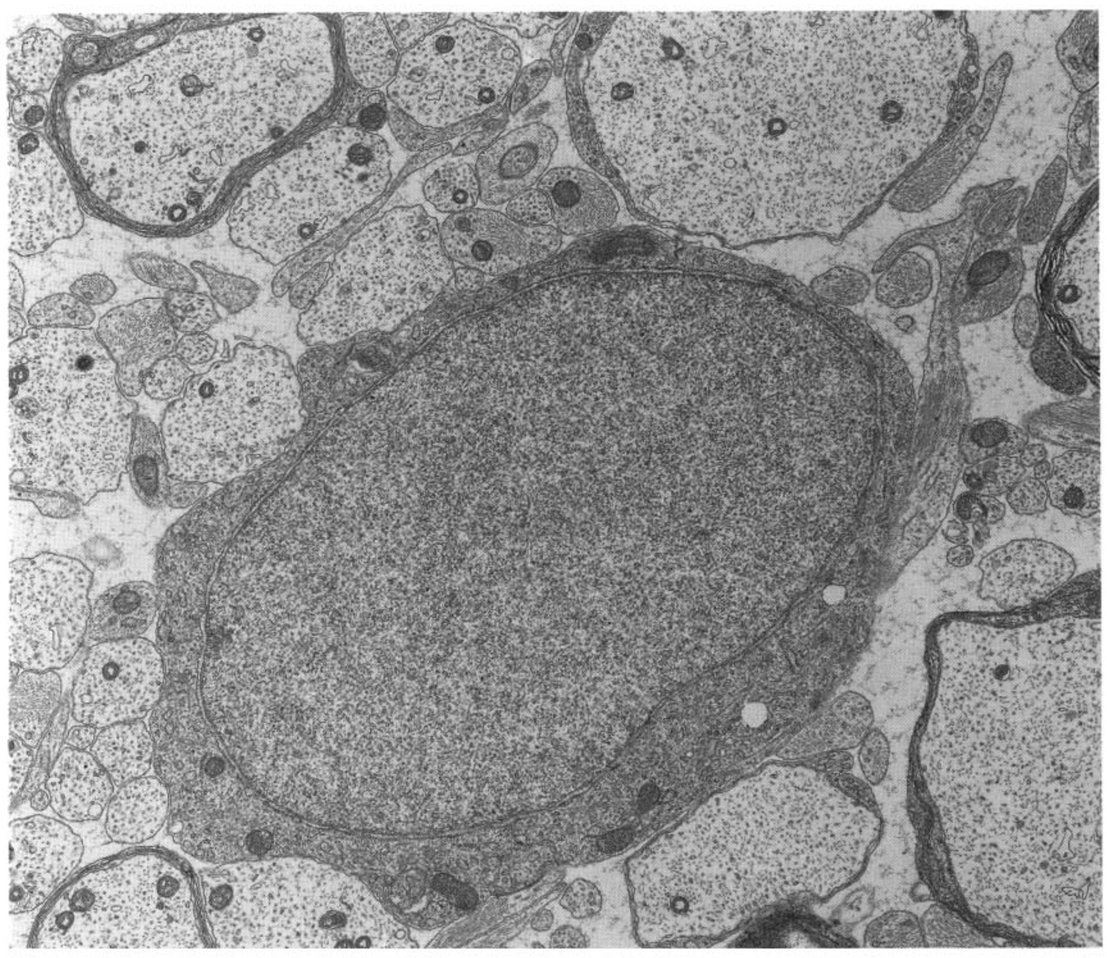


FIG. 66-4. Electron micrograph showing an immature oligodendrocyte in the spinal cord of a 4-week-old shaking pup, which is in contact with a number of nonmyelinated axons. ×11,200.

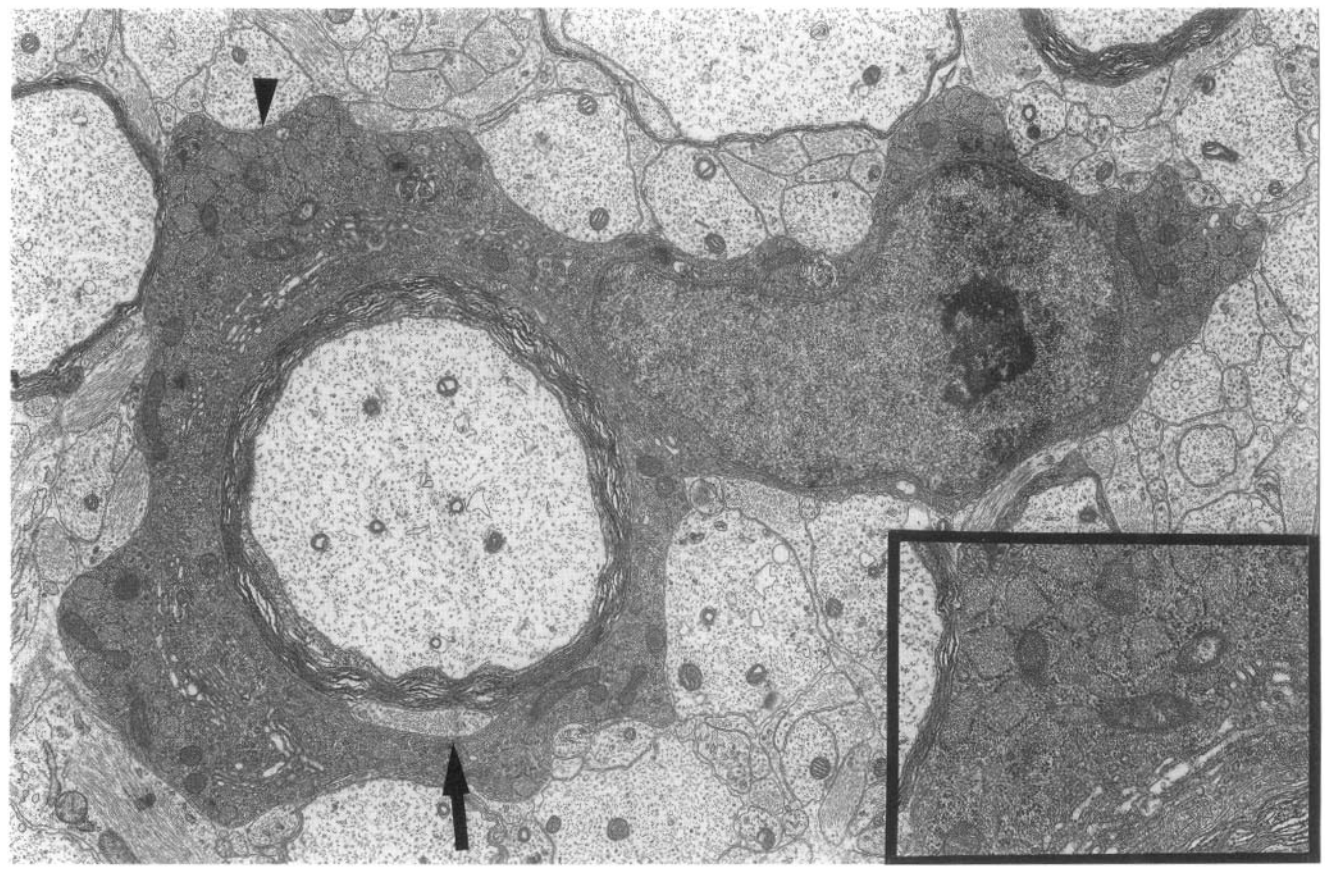
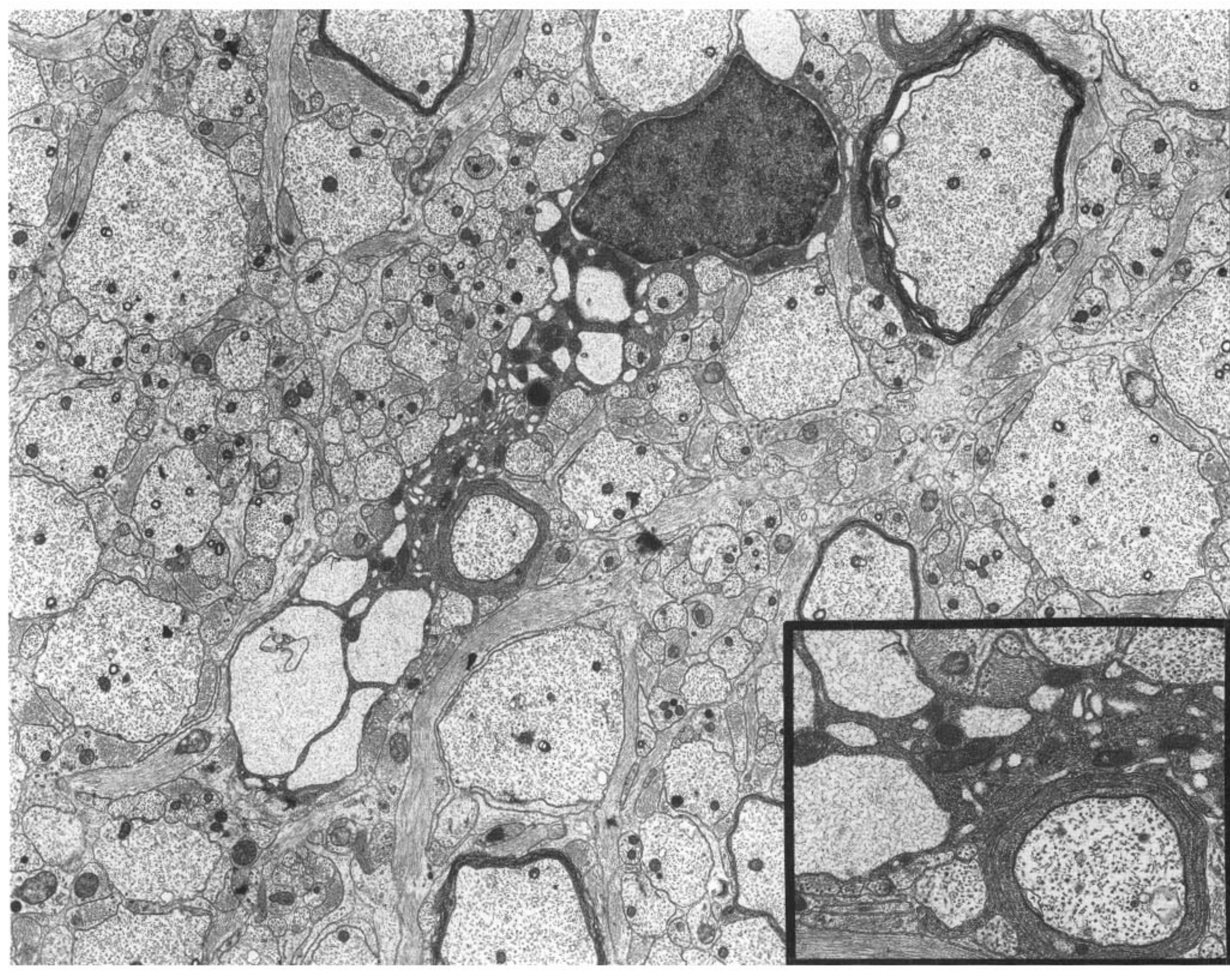


FIG. 66-5. Electron micrographs from the spinal cord of a 4-week-old shaking pup demonstrating distension of the rough endoplasmic reticulum (RER) in oligodendrocytes. **(A)** In this cell the myelin sheath is surrounded by copious cytoplasm with prominent Golgi apparatus and RER (*arrowhead*), which is seen in more detail in the *inset*. An aberrant astrocyte process (*arrow*) is present between the myelin sheath and oligodendrocyte cytoplasm. *Inset*: The RER contains a dark, electron-dense material. ×10,000; **Inset** ×18,500. **(B)** In this oligodendrocyte, swelling of the RER, which contains floccular material, is more marked than the cell in Figure A. This cell may also be myelinating the axon seen in the inset, which has 3 to 4 layers of uncompacted myelin. Many adjacent large diameter axons are unensheathed. ×7150; *Inset*: ×13,400. (*Figure continued.*)

more floccular and there is distention of the perinuclear envelope (Figure 66-5B). A single report of RER swellings in oligodendrocytes in a patient with Pelizaeus-Merzbacher disease (Adachi et al., 1990) suggests this should be more carefully looked at in the future. Several other mutants have been described to have mild RER distension in oligodendrocytes, but this is modest compared to that seen in the *md* rat and *sh pup*. This swelling presumably indicates a failure or slowing of transport of proteins from the RER to the Golgi, perhaps as a result of abnormal folding of the mutant protein. In two of the mutants (*sh pup* and *md* rat) an insertion of a proline into the PLP molecule could result in this transport defect. A similar transport defect may be present in *jp*, where PLP has been demonstrated to be blocked in the Golgi (Rousell et al., 1987), and although the RER does not swell, it is possible that the abnormal protein is rapidly degraded. As of yet, it has not been possible to demonstrate specific accumulation of PLP in the distended RER of the *md* rat or *sh pup*.

More advanced changes in oligodendrocytes, leading to cell death, are common in certain mutants, especially *jp* and the *md* rat. In Figure 66-6, the range of changes found in the *md* rat oligodendrocyte from the massive distention of RER, to margination of nuclear chromatin and finally to pyknosis are seen. The significance of oligodendrocyte death in *sh pup* has not been defined, and it does not appear to be significant in jp^{rsh}. Whether some of this cell death is a result of an increase in programmed cell death (apoptosis) (Barres et al., 1992) or necrosis, remains to be decided.

Proliferation of oligodendrocytes is abnormal in both *jp* (Skoff, 1982; Privat et al., 1982) and the *md* rat (Jackson and Duncan, 1988), but in *jp* there is also evidence of a cell cycle defect as shown by quantitative comparison of cell division and tritiated thy-



B

FIG. 66-5. (*Continued*)

midine labeling (Knapp and Skoff, 1987). Despite an increase in the labeling index in *jp*, there are fewer mitotic cells, and this discrepancy remains to be explained. In contrast, in the spinal cord of jp^{rsh} there is an increase in the number of thymidine-labeled cells compared to controls and a clear increase in glia (M. Fanarraga, personal communication). Although not quantitated, abnormal cell proliferation has also been seen in the *sh pup* (Figure 66-7).

The correlation between oligodendrocyte division, development, function and early cell death, remain as perplexing topics in the X-linked mutants. Careful interpretation of the known facts along with new data may provide important clues, such as the recent finding of early expression of DM-20 mRNA in the 10-day mouse embryo, well before PLP is detectable (Timsit et al., 1992), suggesting that DM-20 may be crucial to the development and survival of the oligodendrocyte. In the *md* rat and *sh pup*, it appears that oligodendrocytes with distension of the RER can ensheathe and myelinate axons, and so a PLP mutation does not totally prevent these oligodendrocyte functions. In jp^{rsh}, it is postulated that the mutation uncouples the defects of hypomyelination and failure of oligodendrocyte development and cell death, which are seen in the other X-linked mutants (Schneider et al., 1992).

Axonal Changes

Axons in many of the X-linked mutants have been found to be abnormal with accumulation of axonal organelles frequently leading to giant axonal spheroids (Barron, 1987) (Figure 66-8). This usually occurs after the myelin defect has become apparent and it is general opinion that these changes are all secondary to the myelin defect as they can also be

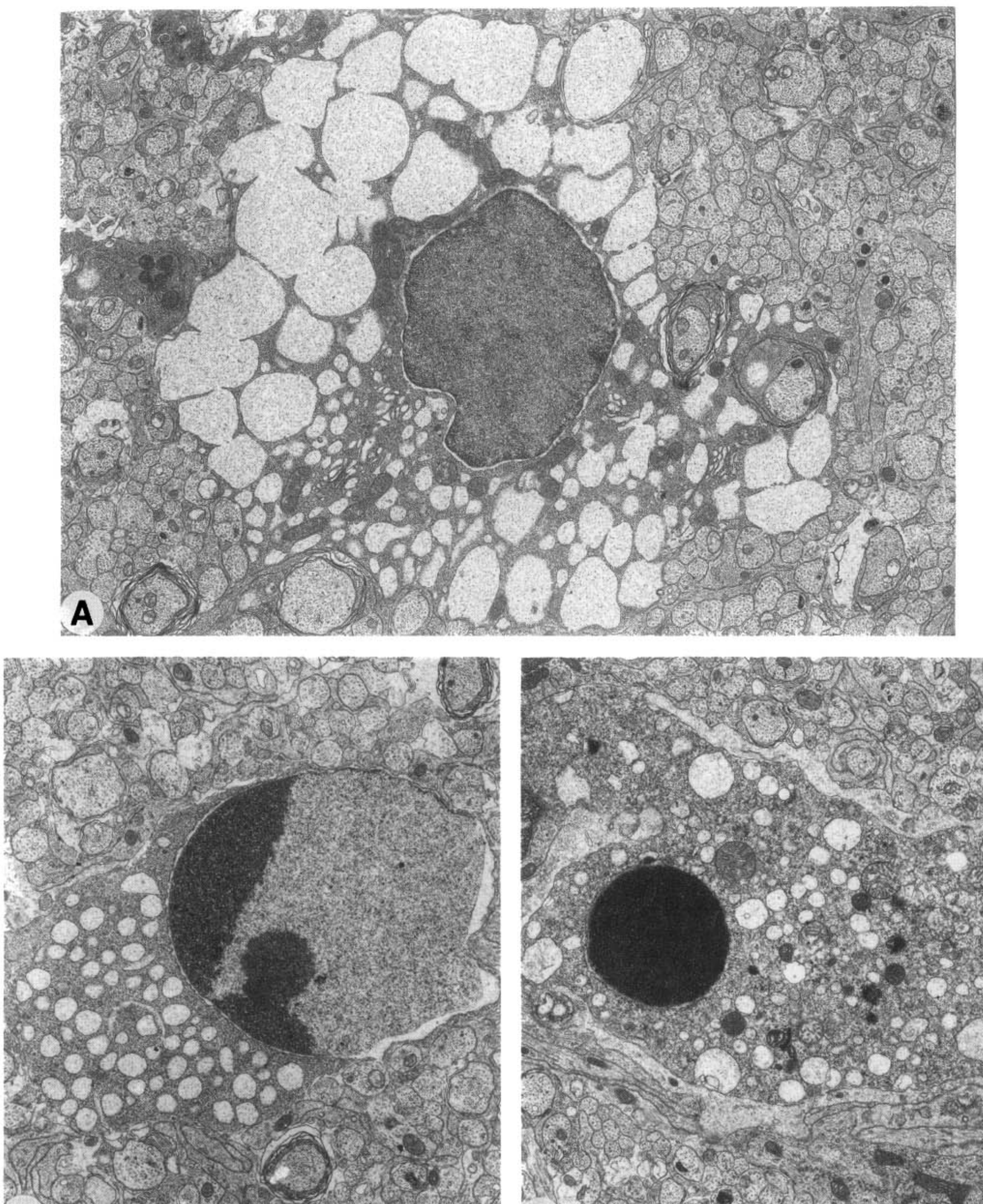


FIG. 66-6. Electron micrographs from the optic nerve of an *md* rat showing the range of oligodendrocyte changes from massive distension of rough endoplasmic reticulum (RER) to cell death. (**A**) The distended RER occupies most of the cytoplasm but the nucleus of the cell is normal. ×9800. (**B**) Early evidence of cell death, where the nucleus has margination of chromatin and the cytoplasm contains distended RER. ×9700. (**C**) A pyknotic oligodendrocyte, which also has swollen RER. ×8800. [From Jackson and Duncan (1988), with permission.]

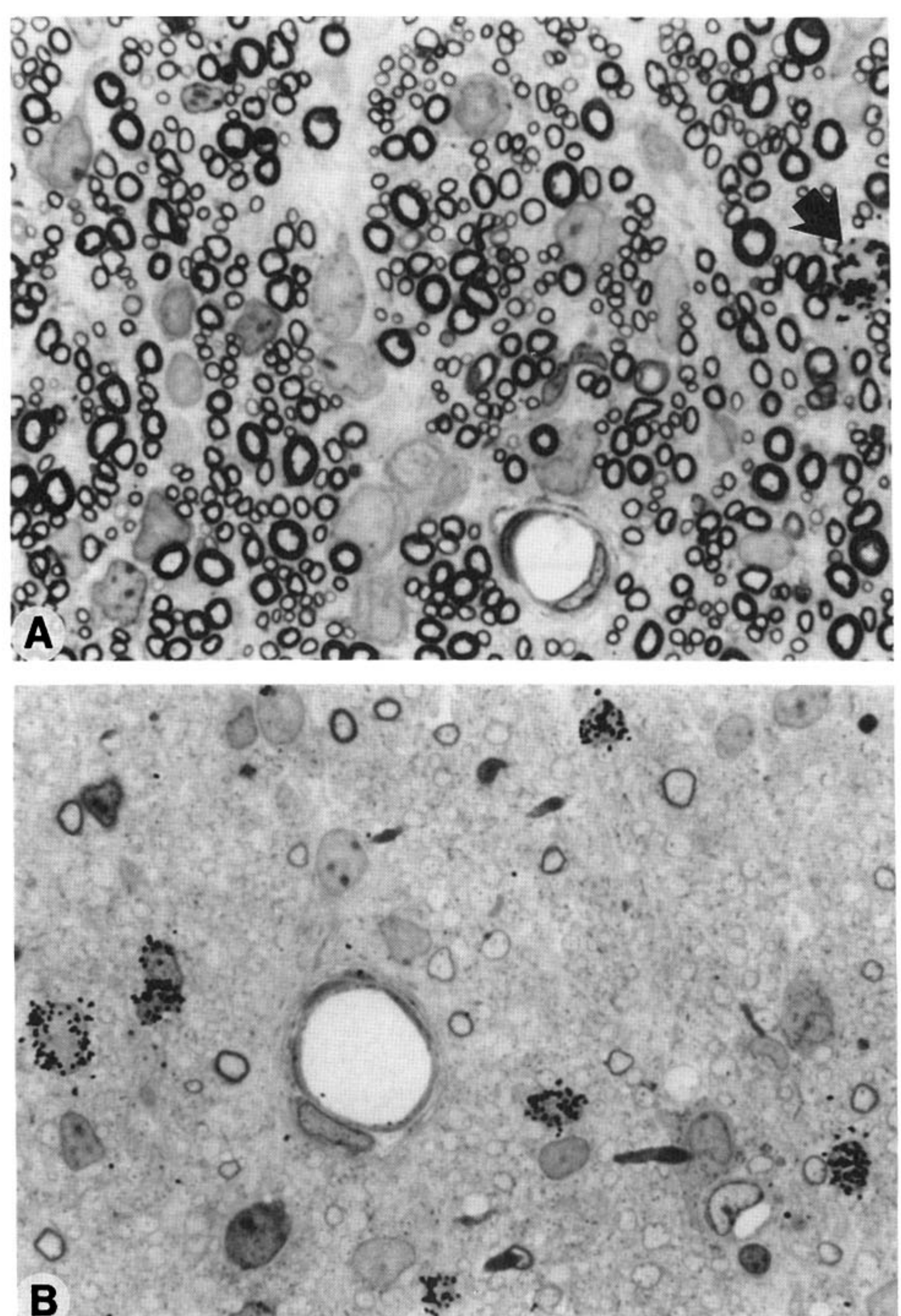


FIG. 66-7. Sections from the optic nerve of (**A**) a 21-day-old normal dog optic nerve and (**B**) an 18-day-old shaking pup. Each dog was pulse labeled prior to sacrifice with triated thymidine. In the normal optic nerve most axons are myelinated and only one labeled cell (*arrow*) is seen. In contrast, in the shaking pup optic nerve, few axons are myelinated and many heavily labeled cells are seen. These cells have the light microscopic appearance of oligodendrocytes. Toluidine blue; ×1100. [From Duncan (1987). *J. Vet. Intern. Med.*, 1:10–23, with permission.]

seen in certain autosomal recessive mutants (*qk* and *shi*). The significance of an increase in proteolysis in retinal ganglion axons of *jp* and jp^{msd} (Nixon, 1982) remains to be resolved. Further information on axons in the X-linked mutants has come from freeze-fracture studies; in the *md* rat patches of E-face particles in the *md* rat adjacent to astrocyte processes have been reported (Waxman et al., 1990). These patches may represent voltage-sensitive sodium channels, involved in conduction of impulses which

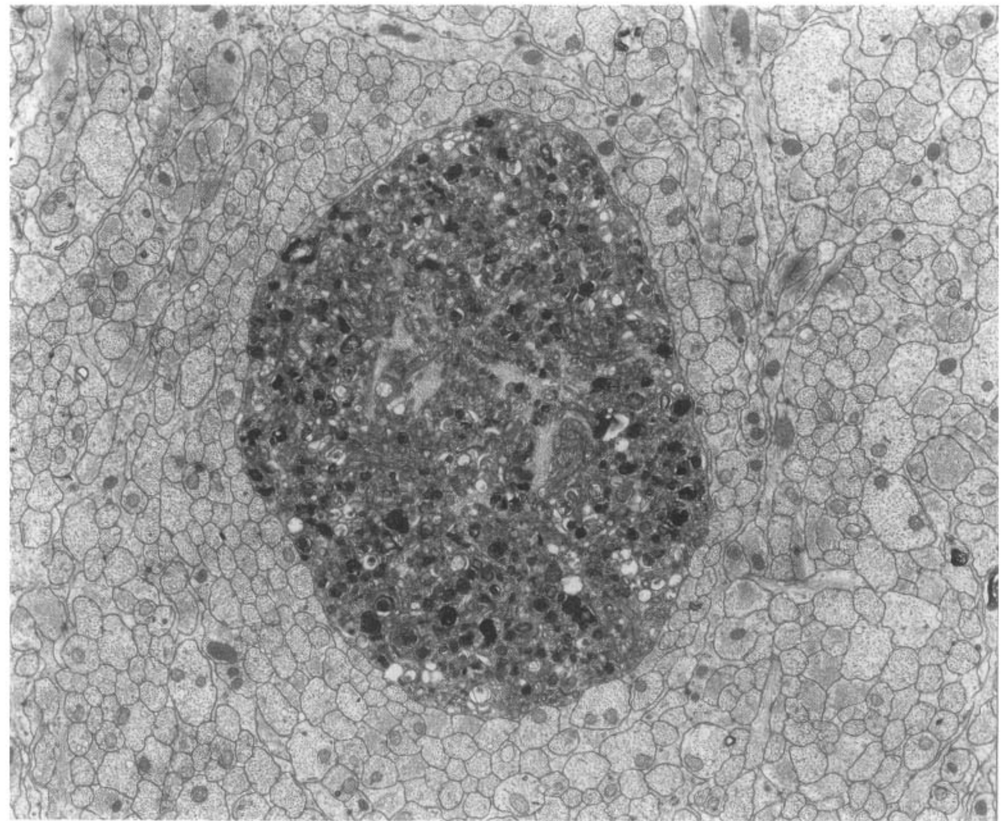


FIG. 66-8. Electron micrograph from the corticospinal tract of an 18-day-old *md* rat. A huge spheroid containing a variety of effete organelles is present. ×8300.

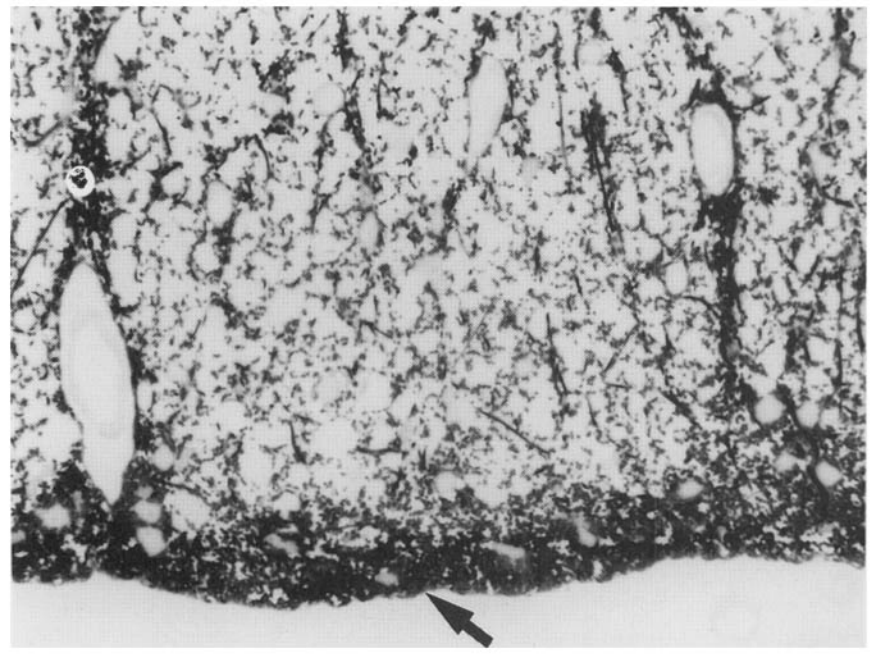
have been shown to occur normally in the *md* rat spinal cord axons (Utzschneider et al., 1992).

Glial cell transplant studies have provided further proof that axons in the md rat, *jp* mouse and *sh pup* can be myelinated, and hence confirming the primary oligodendrocyte defect in these mutants. In addition, quantitative data in the *sh pup* (Duncan et al., 1983) and *md* rat (Dentinger et al., 1985) shows that there are no significant differences in axonal size from controls.

Astrocytes and Microglia

Changes in morphology of astrocytes is a common feature in the X-linked mutants (Baumann et al., 1986). The predominant abnormality is an astrocytic hypertrophy, with an increase in the number of astrocytic processes and glial filaments as seen on electron microscopy and by glial fibrillary acidic protein (GFAP) immunolabeling (Figure 66-9). A more severe gliosis is seen in the mutants with least myelin, that is, in *jp* and *md*, and is least in jp^{rsh}. It has been suggested that this does not relate entirely to myelin loss or absence, as gliosis is seen in spinal cord gray matter of *jp*, and in the central nervous system of the female heterozygotes (Skoff and Knapp, 1992). Freeze-fracture analysis of reactive astrocytes in *md* shows an abnormal development of orthogonal arrays of particles in parenchymal and endfoot processes which may affect normal glial-axonal interactions (Rohlmann et al., 1992).

A prominent astrocytic abnormality in *jp*, *md*, and the *sh pup* is the presence of astrocyte processes between the axon and the myelin sheath (Griffiths and Duncan, 1981; Dentinger et al., 1982; Omlin and Anders, 1983) (Figure 66-10). It is not certain whether this represents aberrant, late oligodendrocyte ensheathement of these astrocytic processes, which frequently abut axons, or the invasion of periaxonal abnormal spaces along the myelin sheath, such as at paranodes (Rosenbluth, 1987). In addition to these structural abnormalities, cultured *jp* astrocytes have been shown to have an abnormal response to high levels of K^+ stimulation and a higher pH than normal astrocytes (Skoff and Knapp, 1992). Whether these alterations in their physiology could result in changes in their putative role in ion



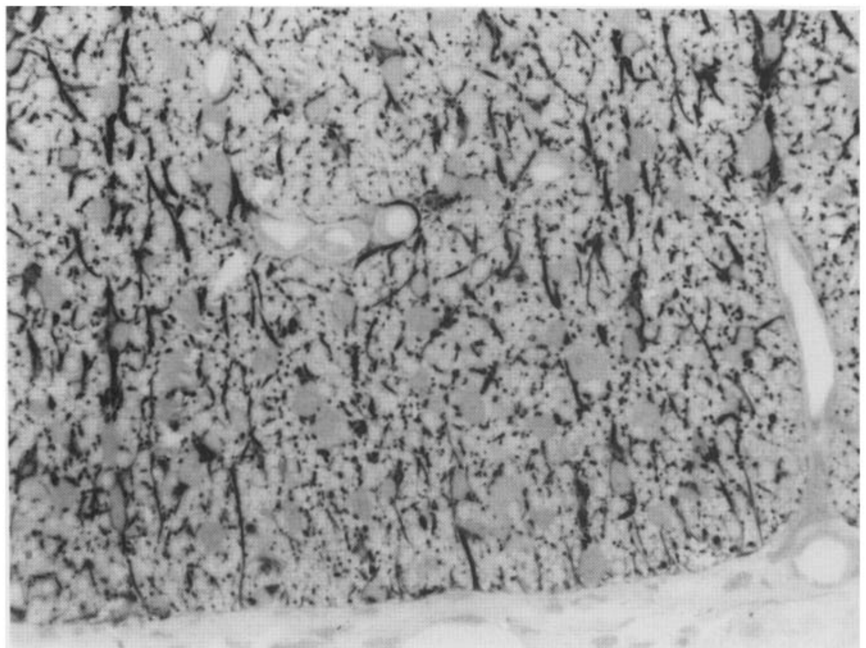


FIG. 66-9. The ventral spinal cord from (**A**) a 13-week-old shaking pup, and (**B**) a normal age-matched control, immunolabeled for glial fibrillary acidic protein (GFAP). In the mutant (Figure A), there is an increase in GFAP activity, both in the subpial area (*arrow*) and in the neuropil. ×500. [From Duncan (1987). *J. Vet. Intern. Med.*, *1*:10–23, with permission.]

exchange or growth factor secretion (e.g., platelet-derived growth factor), remains unknown.

Microglia are numerous in the white matter of both *jp* (Skoff and Knapp, 1992) and the *md* rat (Dentinger et al., 1985; Jackson and Duncan, 1988), especially at the time of maximum oligodendrocyte death in these two mutants. Immunolabeling of microglia in the *md* rat at 20 days of age and in the "aged" strain of *md* (Duncan et al., 1995) at 70 days shows a significant decrease in the older rat (Duncan and Li, unpublished data) corresponding to the reduced oligodendrocyte cell death at older ages.

HETEROZYGOTES

The female heterozygote carriers of the X-linked myelin mutants have the potential to demonstrate myelin mosaicism in the central nervous system as a result of the process of random X-linked inactivation

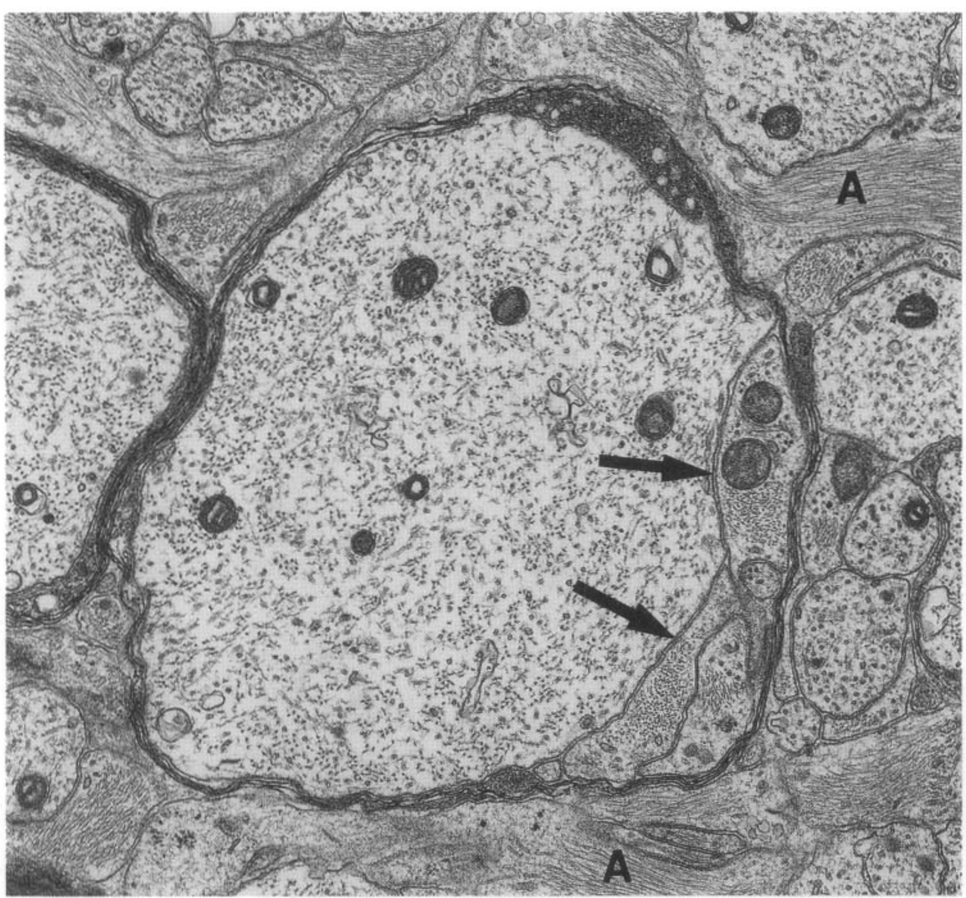
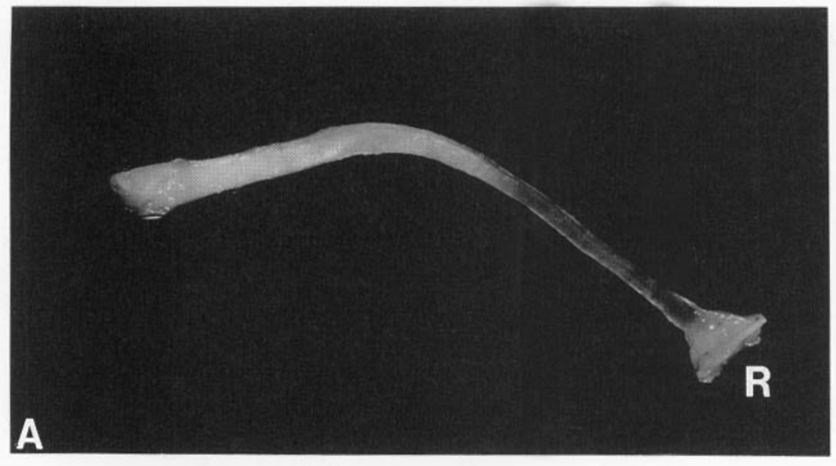
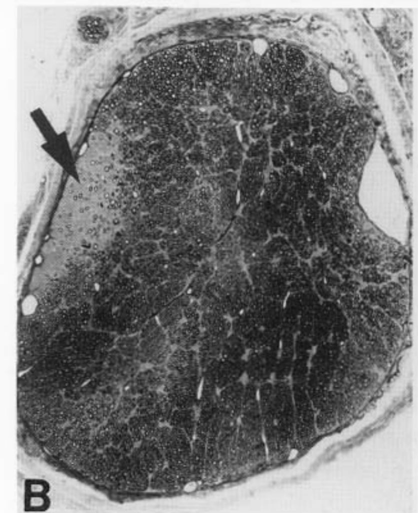


FIG. 66-10. Electron micrograph from the spinal cord of a 4-week-old shaking pup. A large diameter axon which has (2 to 3) lamellae has three astrocyte processes (*arrows*) interdigitated between the axon and the thin myelin sheath. Astrocyte processes (*A*), packed with glial filaments are obvious in the neuropil. ×17,900.

(Lyon, 1972). Such mosaicism was first described in the optic nerve of the *jp* mouse heterozygote (Skoff and Montgomery, 1981), and has since been described in the same structure in the *md* rat and *sh pup* (Duncan et al., 1987a). In the *md* rat, a detailed study of the optic nerves has shown that certain nerves show a gross deficiency in myelination, (Figure 66-11), which is the result of either absence of myelin, or a mosaic pattern consisting of adjacent patches of myelination and nonmyelination (Figure 66-11) (Duncan et al., 1993). A semiquantitative analysis of the distribution of the myelin defect in young and old *md* heterozygotes, has shown that the retinal end of the nerve is more severely affected (Duncan et al., 1993), providing further indirect evidence supporting the theory of longitudinal migration of the oligodendrocyte progenitor in the optic nerve (Small et al., 1987) as opposed to its radial spread. In *jp*, *md*, and the *sh pup*, mosaicism is more marked in the optic nerve than in any other areas of the central nervous system. Patches of nonmyelination or hypomyelination in the spinal cord have only been seen in the *sh pup* and *md* rat (Duncan, unpublished). In *jp* a more diffuse hypomyelination is present in the spinal cord (Bartlett and Skoff, 1986) and anterior commissure (Rosenfeld and Friedrich, 1984), which compensate with time (Bartlett and Skoff, 1986; Rosenfeld and Friedrich, 1986). Differences in cell death and proliferation in *jp* heterozygotes from affected males have been summarized by Skoff (Skoff and Knapp, 1992). The apparent predilection for mosaicism in the optic nerve therefore suggests that it has a different pattern of oligodendrocyte progenitor migration and population from the remainder of the central nervous system, or there is a limit in the ability to recruit normal cells into the optic nerve for repair purposes.

The findings in jp^{rsh} differ from the observations described above (Fanarraga et al., 1991). Optic nerves of female carriers show no amyelinated patches or hypomyelination, and in the spinal cord, only scattered hypomyelination is seen. Occasional





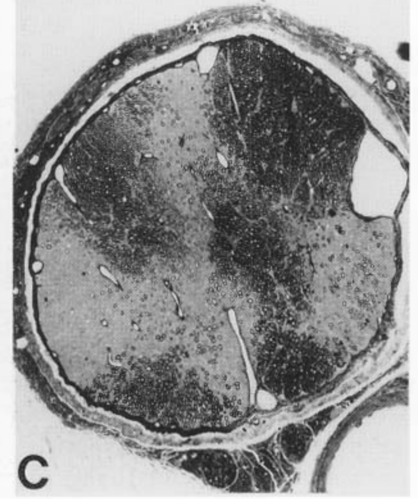


FIG. 66-11. (A) The whole optic nerve from a 14-month-old *md* rat heterozygote. The retinal end (*R*) of the nerve is transparent indicating a total absence of myelin in contrast to the chiasmal end of the nerve. (B and C) Sections from different areas of the same optic of 6-month-old known heterozygote. In the section nearer to the chiasm (Figure B), only a small patch of nonmyelination (*arrow*) is seen. Closer to the retina (Figure C), an obvious mosaic pattern of myelin and nonmyelination can be seen. The myelinated areas have well circumscribed boundaries. Toluidine blue; Figure A: ×7; Figures B and C: ×187. [Modified from Duncan et al. (1993) with permission.]

myelinated fibers which are MBP-positive and PLP-negative as determined by immunolabeling are seen in the spinal cord (Fanarraga et al., 1991).

Affected female rats born to known *md* heterozygotes have been found in three *md* rat colonies (Rosenbluth et al., 1990; Koeppen et al., 1992; Duncan, unpublished data). These female rats have an identical phenotype to the hemizygote males and examination of their karyotype has shown that they have X-monosomy.

AUTOSOMAL RECESSIVE MYELIN MUTANTS

This group of mutants is more heterogeneous and fewer in number than the X-linked mutants. Four of these, the shiverer (*shi*) mouse and its allele shi^{mld}, the quaking (*qk*) mouse, and the *taiep* rat are described together and mention made of other less well characterized mutants of autosomal recessive nature, where hypomyelination is seen. A summary of the abnormalities in these mutants is outlined in Table 66-2.

Amount of Myelin and Myelin Structure

The myelin deficit seen in all of these mutants is not as marked as seen in the most severely affected X-linked mutants. Comparison of quantitative data on the amount of myelin in *shi* (Billings-Gagliardi et al., 1986) and *qk* (Nagara and Suzuki, 1981) suggests that there is considerably less myelin in the central nervous system of *shi*, although the quantitative methods were different in each study. However, it has been estimated that *shi* optic nerve has about 20% of controls (Billings-Gagliardi et al., 1986), whereas *qk* optic nerve has 60% (Friedrich, 1974). In *qk*, the myelin defect is most marked in the initial stages of myelination and there is a steady increase in myelin with time (Nagara and Suzuki, 1981). A notable difference between *shi* and shi^{mld} has been reported. The latter lives for slightly longer than *shi* and this has been associated with an increasing amount of myelin, with the presence of MBP in older shi^{mld} mice (Matthieu et al., 1986). Subtle differences in the amount of myelin and its ultrastructure, and indeed in the survival time of shi^{mld} mice derived

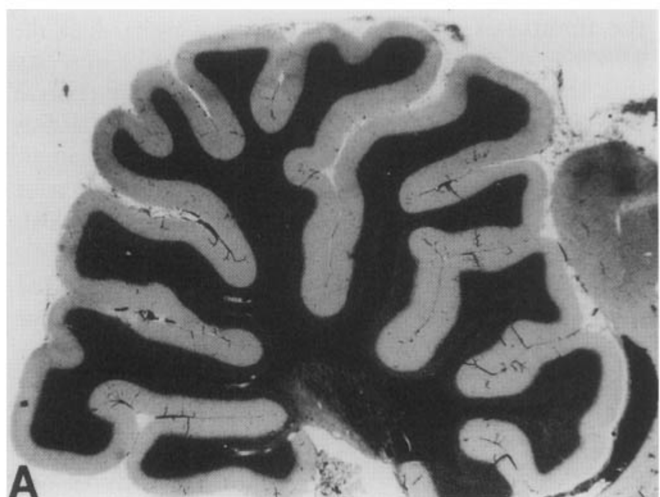
TABLE 66-2. *Summary of the Phenotypic Abnormalities in the Autosomal Recessive Central Nervous System Myelin Mutants*

Phenotype	shi	shi^{mld}	qk	taiep
Amount of myelin	+	++ but ↑ with time	++ Hypomyelination ↑ with time	+++ Hypomyelination but then progressive demyelination
Myelin structure	neg MDL	pos/neg MDL	Abnormal radial component	Normal
Myelin immunolabeling	MBP-neg	MBP-neg (with time some myelin MBP-pos)	NAD	MBP and PLP-pos
Oligo number	↑	?	↑	?↑
Oligo structure	Initially ↑ vacuoles	Initially ↑ organelles ↑ microprocesses	↑ Membranous profiles in young mice	Progressive microtubular increase
Oligo proliferation	NE	NE	NE	?
Cell death	NAD	NAD	NAD	NAD
Gliosis	NAD	NAD	+	?
Microglia	NAD	NAD	NAD	NAD

MDL, major dense line; MBP, myelin basic protein; PLP, proteolipid protein; Oligo, oligodendrocyte; NAD, no abnormality detected; NE, not examined; +, severe reduction in myelin; ++++, normal.

from different backgrounds, have been identified, and this should be kept in mind when comparisons are made (Shen et al., 1985). In the *taiep* rat, myelination appears to commence normally, although myelin sheaths may be thin, but the major abnormality appears later in life with the progressive loss of myelin from more rostral structures such as the corpus callosum, cerebellum, and optic nerves (Duncan et al., 1992) (Figure 66-12). In the spinal cord of older *taiep* rats is a diffuse loss of myelin in the lateral and ventral white matter. However, the most striking deficit is in the fasciculus gracilis and corticospinal tracts, where by 12 months of age, few axons are myelinated (Figure 66-13).

In all three mutants the myelin sheaths axons are thin, although in *taiep*, many axons have a normal myelin sheath. In both *shi* and *qk*, the myelin formed is often poorly compacted and in *shi* the myelin formed lacks a major dense line. In *shi^{mld}* however, the increase in myelin is associated with the presence of a major dense line in thicker myelin sheaths, a result of the later deposition of MBP in *shi^{mld}* myelin



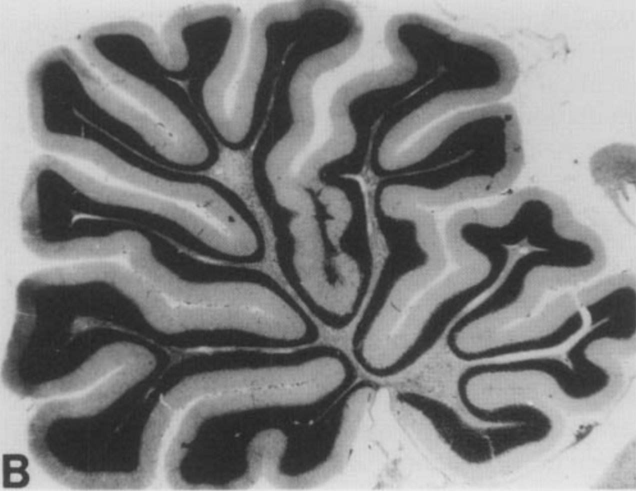


FIG. 66-12. The cerebellum from (A) a 12-month-old normal and (B) *taiep* mutant rat. A lack of myelin can be noted in the cerebellar white matter and brainstem of the *taiep* rat. Heidenhain stained; ×20.

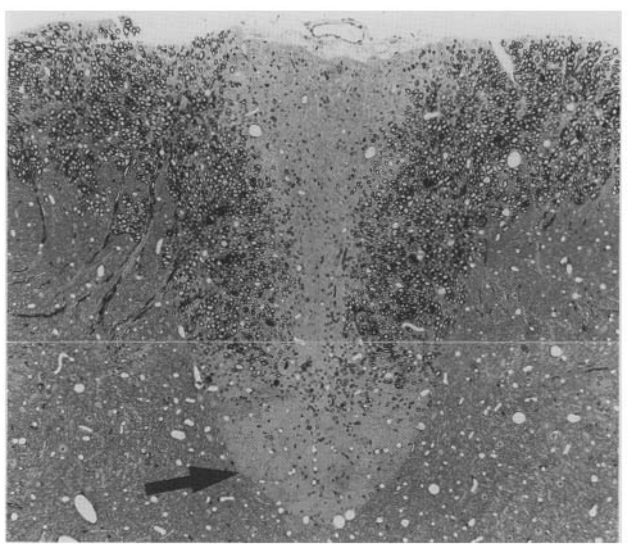


FIG. 66-13. The dorsal columns from a 12-month-old *taiep* rat. The fasciculus gracilis and the corticospinal tract (*arrow*) have almost no myelin present, compared to the adjacent fibers of the fasciculus cuneatus. Toluidine blue; ×160. [Modified from Duncan et al. (1992), with permission.]

(Mikoshiba et al., 1992). As the *qk* mouse ages, myelin sheaths become thicker, and there is much less evidence of aberrant myelin formation as is seen in young *qk* mice, suggesting that *qk* mice oligodendrocyte are eventually capable of forming normal compact myelin (Nagara and Suzuki, 1981). *Taiep* myelin in contrast, appears to have a normal ultrastructural appearance (Duncan et al., 1992).

The peripheral nervous system of *shi* and *qk* have also been examined in detail. In *shi*, despite the total lack of MBP, the structure of myelin in the peripheral nervous system is almost normal, with no hypomyelination and a normal intraperiod line. In *qk*, earlier work had suggested that hypomyelination was seen in the peripheral nervous system (Samorajski et al., 1970), but a morphometric study of myelin sheath thickness and internode length has shown that *qk* internodes are very short and that the myelin sheaths have an appropriate diameter (Beuche and Friede, 1985). Initial analysis of the *taiep* peripheral nervous system indicates that myelination is normal.

Oligodendrocyte Number and Abnormalities

Unlike the X-linked mutants which overall have a reduction in the number of mature oligodendrocytes (with the exception of jp^{rsh}), *shi*, *qk*, and *taiep* have all been reported to have more oligodendrocytes than normal (Friedrich, 1975; Nagara and Suzuki, 1981; Duncan et al., 1992). This has been most critically evaluated in *qk* (Friedrich, 1975; Nagara and Suzuki, 1981), where a two- to fourfold increase has been reported in the optic nerve and in the spinal cord (Friedrich, 1975). In *taiep*, there appears to be a generalized increase in oligodendrocytes throughout the central nervous system, but this has to be confirmed by detailed morphometry. Quantitation of oligodendrocyte number can be inaccurate because of the differences in the area of the white matter between mutant and wild type (Friedrich, 1975). In addition, the identification of either mature or immature oligodendrocyte is difficult at the light and electron microscopic levels, and immunolabeling may be required. If an increase in oligodendrocyte numbers is proven in these mutants (already shown in *qk*), this may provide an opportunity to study the mechanisms behind this, that is, whether this results from increased cell division or survival, with fewer oligodendrocyte progenitors undergoing programmed cell death (Barres et al., 1992). If the increase is due to abnormal cell division, it should be determined whether this results from persistent nonmyelination.

In none of these mutants has an increased oligodendrocyte cell death been reported, but cytoplasmic changes in the oligodendrocytes in each have been documented. In *shi*, between 2 and 4 weeks of age, oligodendrocytes have prominent vacuoles and vesicles, but these changes are not seen in mature mice (Inoue et al., 1983). Similar abnormalities have been reported in shi^{mld} (Matthieu et al., 1986). A prominent ultrastructural abnormality seen in *shi* is the collection of oligodendrocyte microprocesses (Shen and Billings-Gagliardi, 1985). This has also been reported in the *taiep* rat (Duncan et al., 1992) and may reflect abnormalities of the oligodendrocyte cytoskeleton. In neonatal *qk*, there is a prominent dilatation of smooth endoplasmic reticulum and vesicle proliferation, but these changes are not seen in older *qk* mice (Nagara and Suzuki, 1981).

The *taiep* rat has the most unique oligodendrocyte cytoplasmic abnormality, with marked accumulation of microtubules throughout the oligodendrocyte cytoplasm (Duncan et al., 1992). The microtubules frequently become associated with smooth endoplasmic reticulum (Figure 66-14). The first signs of microtubule accumulation is seen at about 1 month of age and progresses with age. In the older rats, evidence on myelin breakdown is seen on electron microscopy (Figure 66-14). This mutant provides a model in which to define the critical role of the microtubular network in myelination and myelin maintenance. If a progressive accumulation of microtubules is the primary defect in *taiep*, the myelin defect may result from a disturbed and later deranged transport of myelin proteins within the oligodendrocyte.

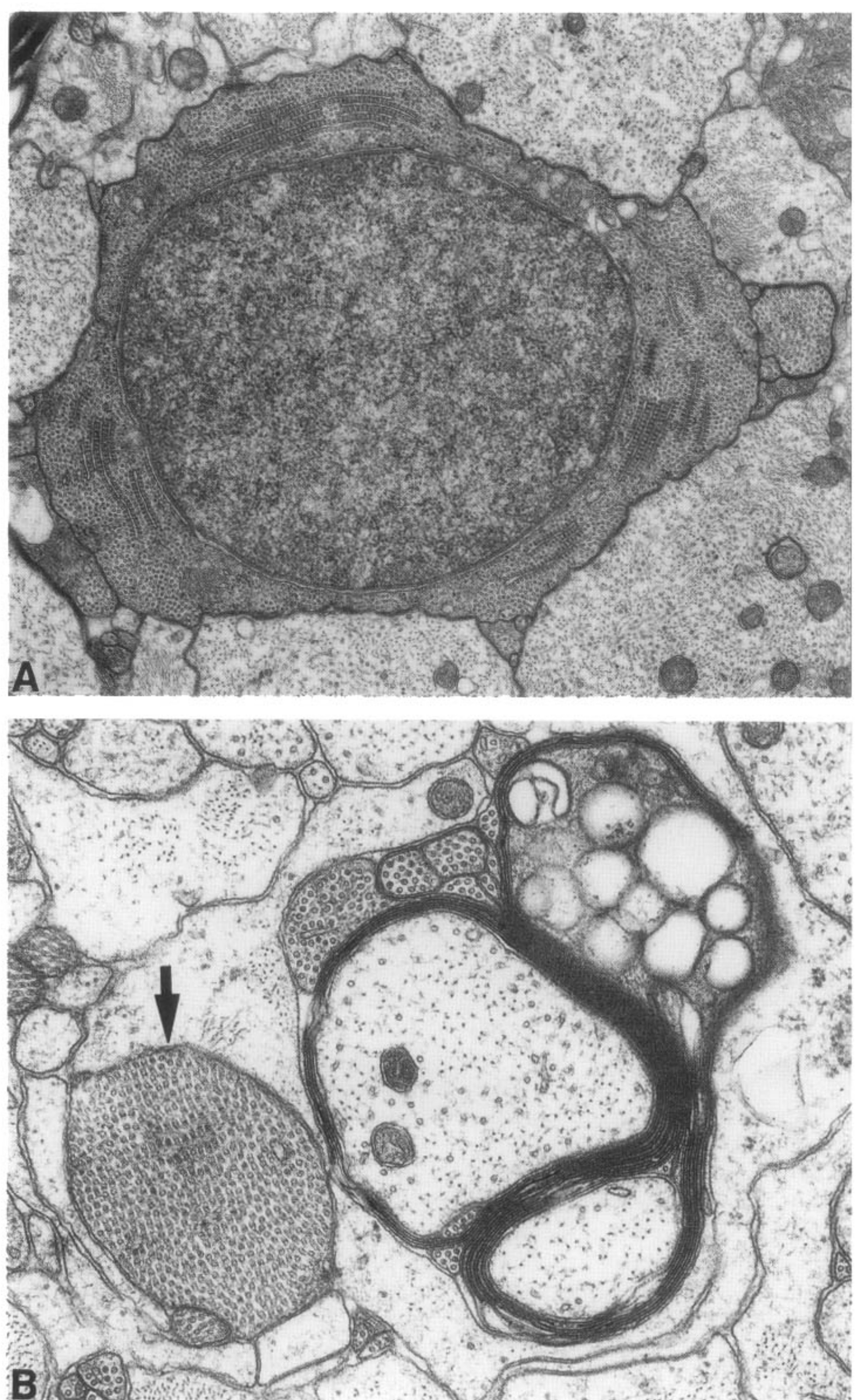


FIG. 66-14. Electron micrographs from a 9-month-old *taiep* rat. (**A**) The oligodendrocyte has an increased number of microtubules, some of which run in parallel arrays along smooth cisternae. (**B**) The myelinated fiber present has an aberrant myelin sheath, which is starting to break down, and has a duplicated outer mesaxon. An adjacent oligodendrocyte process (*arrow*) is packed with microtubules. Figure A: ×18,700; Figure B: ×40,500. [Modified from Duncan et al. (1992), with permission.]

Other Glial and Axonal Changes

In general, an astrocytic response to the primary glial abnormality is not a marked feature in these mutants. In fact, there is only a mild gliosis in *qk* and no evidence of this has been emphasized in *shi*. The *taiep* rat shows moderate ultrastructural evidence of gliosis but immunocytochemistry for GFAP has yet to be performed. Quantitation of GFAP by Western blot analysis may be the most accurate way to determine the astrocytic response in these mutants (Norton et al., 1992).

Unlike the X-linked mutants there is no evidence of a microglial proliferation. Likewise, there are few significant changes in axons with only occasional axonal spheroids reported in the *qk* cerebellum and a moderate number in the *taiep* central nervous system. However, more extensive vacuolar degeneration of nerve roots, similar to that seen in the aging peripheral nervous system has also been described in *qk* (Suzuki and Nagara, 1982). The possibility of abnormal interactions between axon and oligodendrocytes in these mutants has been suggested in a report of sodium channel density in the *shi* mouse (Noebels et al., 1991). In the *shi* brain, there is a marked excess of sodium channels, suggesting a failure of downregulation of sodium channel density in *shi* axolemma as a result of an abnormal cell-cell interaction (Noebels et al., 1991).

OTHER AUTOSOMAL RECESSIVE MUTANTS

A number of other less well-known myelin mutants have been reported, which cannot be discussed in detail here, but which are summarized for completeness. Two murine mutants have been described, the Syrian mutant hamster (Nunoya et al., 1985; Kunishita et al., 1986) and the zitter rat (Kondo et al., 1991; Kondo et al., 1992), which show mild hypomyelination. The hamster shows a delay in myelination of the central nervous system, but with all myelin proteins being present in the myelin formed (Kunishita et al., 1986). The zitter rat shows more pronounced hypomyelination in the spinal cord than the hamster, which persists to 37 weeks of age. Accumulation of abnormal membranous structures and a distension of the RER, perinuclear cisternae, and Golgi in oligodendrocytes are the most prominent feature of this mutant, and may indicate a defect in membrane biosynthesis. All the major myelin proteins are present in zitter myelin. A very recent report of a severe myelin deficiency in a strain of Long Evans rats appears to offer new opportunities to explore the myelination process (Delaney and Kwiecien, personal communication). This rat has no myelin in the central nervous system as seen on light microscopy, yet lives to 3 to 5 months of age. Preliminary ultrastructural studies suggest premature oligodendrocyte death and abnormal oligodendrocyte development (Duncan, unpublished data).

In the dog, two breeds have been found to have an inherited defect which results in a delay in myelination of the peripheral lateral and ventral white matter of the spinal cord (Vandevelde et al., 1978; Kornegay et al., 1987). This may result from a delay or failure of migration of oligodendrocyte progenitors to these sites (Duncan, unpublished information). Finally, dysmyelination has been reported in the central nervous system in certain storage disorders, including caprine β-mannosidosis (Lovell and Boyer, 187) and canine GM1 gangliosidosis (Kaye et al., 1992).

CONCLUSION

New mutants with defects in other myelin proteins, such as MAG or CNP, are eagerly sought, as is the final definition of the *qk* mutation. As with P_0 in the peripheral nervous system (Giese et al., 1992) gene knockout strategies, ablating expression of MAG and CNP, may confirm their postulated roles in myelination. Indeed new mutants may lead to the finding of undescribed central nervous system proteins such as was the case of peripheral myelin protein-22 in the peripheral nervous system and the *trembler* mutant (Suter et al., 1992). Despite the many recent advances, a complete understanding of the myelination process is still elusive, and myelin mutants will continue to play a pivotal role.

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67 Viral infections of neuroglial cells

BY MONIQUE DUBOIS-DALCQ

Viruses that spread and replicate in the nervous system are often called neurotropic because they can make their way to nervous tissue where they find specific cellular receptors for binding and entry and a milieu favorable to their replication. In this chapter I will describe how certain viruses infect neuroglial cells—oligodendrocytes, astrocytes, microglia and ependyma in the central nervous system and Schwann cells in the peripheral nervous system—and how these infections cause experimental and human neurological diseases. Viruses can indeed affect neuroglial cell survival and functions, leading to neuropathological changes such as demyelination, chronic inflammation, and even neurodegeneration. First, I discuss common properties and mechanisms underlying viral diseases of neuroglial cells. Then I review selected examples of such viral diseases and how their molecular pathogenesis is currently being studied.

Are Neuroglial Cells Susceptible to Many Different Viruses and Is Infection of Neuroglial Cells Selective?

Both DNA and RNA viruses, including retroviruses, can infect neuroglia in animal and man. The names, families, and main structural properties of these viruses are summarized in Table 67-1, which may serve as a guide to the abbreviations of virus names used throughout this chapter. Several of these viruses can also replicate in neurons, but lentivirus and human polyoma virus expression appears to be truly restricted to neuroglia. A few important viruses that are pathogenic in humans show a tropism selective for neurons, such as rabies virus, which replicates in many central nervous system neurons; the human poliovirus, which selectively infects spinal cord motoneurons; and latent forms of herpes simplex virus types 1 and 2 (HSV-1 and -2), which reside in sensory ganglia neurons (for reviews see Johnson, 1982; Kristensson and Norrby, 1986; Fields et al., 1990).

How Do Neurotropic Viruses Spread to the Brain?

First, a virus can spread directly to a neural site such as the olfactory epithelium (coronaviruses; it is not clear that this route is ever used in natural infections) (Lavi et al., 1988), the sensory nerve terminals in the skin or mucosa (HSVs), or the neuromuscular junction (rabies, polio?) (Ren and Racaniello, 1992). Viruses can also disseminate from the blood to the brain by passing through the fenestrated endothelium of the choroid plexuses to the cerebrospinal fluid or by traversing and/or replicating in brain endothelial cells (mouse retrovirus, Cas BrE, see below). Finally, viruses can bypass the tight junctions between brain endothelial cells, crossing the blood-brain barrier inside blood mononuclear cells (T cells, B cells, or monocyte-macrophages), a situation frequently seen with viruses that infect both the immune and the nervous systems (lentiviruses, HTLV-1, measles virus, JC virus).

Have Receptors for Neurotropic Viruses Been Characterized?

The human poliovirus receptor has been characterized both functionally and structurally; it is a member of the immunoglobulin superfamily (Mendelsohn et al., 1989). Mice that do not express the poliovirus receptor and are normally not susceptible to infection with human poliovirus can be made susceptible by transferring the poliovirus receptor gene to the mouse germline (Ren and Raccaniello, 1990). When transgenic mice expressing this receptor in several organs including the nervous system were infected with human poliovirus, virus replicated only in the brain, spinal cord, and muscle, indicating that viral tropism is governed not only by expression of the poliovirus receptor gene but also by host cell factors (Ren and Racaniello, 1992). It is not known yet whether the receptor for the mouse picorna viruses that cause a demyelinating disease (see the section *Infections With Theiler's Murine Encephalomyelitis Virus* below) is related to the poliovirus receptor.

Although much progress has been made recently in the characterization of other viral receptors, their role in infections of the nervous system is not yet clearly established. In addition, viruses may be able to use alternate receptors for their entry or may need

TABLE 67-1. *Viruses Infecting Neuroglial Cells*

Virus Families	Structural Properties of the Viruses	Specific Virus Names	Cell Type Infected	Mode of Propagation	Receptor Known?
I RNA viruses					
Picornaviridae	Nonenveloped icosahedral symmetry	Poliovirus	Anterior horn neurons and some brain nuclei	Intestinal tract → blood → brain to muscle?	Poliovirus receptor (humans)
	28–30 nm 1SS RNA ~8 kb	Theiler murine encephalitis virus (TMEV)	Neurons first; astrocytes and oligodendrocyte later		?
Togaviridae	Enveloped, with fine peplomers 60–70 nm 1SS RNA ~12 kb	Pestivirus Border disease in sheep	Oligodendrocytes *in vivo* and *in vitro*; astrocytes and neurons also *in vitro*	*In utero*	?
Coronaviridae	Enveloped, with club-shaped spikes Helical nucleocapsid	Corona virus A59 in mice,		Intestinal tract, blood, olfactory neurons, endothelial cells?	Carcinogenic embryonic antigen (CEA) variants; accessory molecule?
	80–160 nm (mouse:90) 1SS RNA, 16 to 31 kb	JHM in rats and mice			
		229E in humans		Respiratory tract	Aminopeptidase
Paramyxoviridae	Enveloped, large peplomers, Helical nucleocapsid, Pleomorphic, 150–300 nm	Measles virus (MV) (subacute sclerosing panencephalitis) (SSPE)	Neurons, astrocytes, and oligodendrocytes	Blood brain, in lymphocytes and monocytes-macrophages?	Neuraminic (sialic acid)
	1 negative sense SS RNA, 16–20 kb	Canine distemper virus (CDV)	Astrocytes and oligodendrocytes		
II DNA viruses					
Papovaviridae: Polyomaviruses	Nonenveloped icosahedral symmetry, 45 nm, circular dsDNA, 5 kb	JC virus (JCV, human) (progressive Multifocal leukoencephalopathy)	Oligodendrocytes and astrocytes	Bone marrow → circulating white cells → brain; from genitourinary tract?	?
		Simian virus 40			? Class I molecules
Herpesviridae	Enveloped, spikes, icosahedral nucleocapsid, ~150 to 200 nm 1ds DNA 120–200 kb	Herpes Simplex Virus type 1 and 2 (HSV-1 and 2)	Latency in sensory neurons; Schwann cells, astrocytes, and oligodendrocytes	Skin and genital mucosa → sensory nerves and ganglia. Trigeminal and olfactory tracts → CNS	Heparin sulfate proteoglycan; accessory molecule?
		Varicella zoster (VZV)	Latency in sensory neurons; Schwann cells	Skin and mucosa → sensory nerves and ganglia	
		Cytomegalovirus (CMV)	Ependymal cells, other neuroglial cells?	Congenital infection; blood → brain, in monocytes.	
III Retroviruses					
Type C oncoviruses	Enveloped, spikes; icosahedral/helical nucleocapsid 80 to 130 nm	CasBrE ecotropic retrovirus (Lake Casitas wild mouse)	Cerebellar neurons; microglia; endothelial cells	Blood viremia → brain	?

(*Table continued on next page*)

TABLE 67-1. *(Continued)*

Virus Families	Structural Properties of the Viruses	Specific Virus Names	Cell Type Infected	Mode of Propagation	Receptor Known?
Lentiviruses	2 SS −9 kb RNAs Reverse-transcription → integration of	Visna virus (sheep)	Choroid plexuses, macrophages and oligodendrocytes??	Blood → brain, in T cells and monocytes-macrophages?	50K protein?
	proviral DNA → RNA transcripts	Human and simian immunodeficiency viruses (HIV-1, SIVS)	Microglia; immature fetal astrocytes; Oligodendrocytes??	Blood → brain, in T cells and monocytes-macrophages?	CD4 galactocerebroside?
Oncovirinae		Human T cell leukemia virus (HTLV-1)	Macrophages, microglia? T cells?		?

Compiled from information in *Fields Virology* (1990), Dubois-Dalcq et al. (1984) and references quoted in the text. It should be noted that this is not an exhaustive list of all viruses that infect neuroglial cells but rather a series of selective and well-known examples of such infections.

accessory molecules only found in certain cell types. In enveloped viruses, it is always a major viral glycoprotein extracellular domain that contains the specific recognition sites for cellular receptors. The receptor for mouse hepatitis virus (MHVR1) is recognized by the S protein of these coronaviruses and is a member of the carcinoembryonic antigen (CEA) family of glycoproteins, which are also part of the immunoglobulin superfamily (Williams et al., 1991). Although several strains of MHV specifically infect neuroglial cells (see below), one type of transcripts for MHVR1 present in liver and gut—mmCGM1—is not significantly expressed in the brain of susceptible mice. However, a splice variant—mmCGM2—is found in the central nervous system, suggesting that MHV may utilize different CEA molecules in the liver and in the brain (Yokomori and Lai, 1992a). In addition, mouse coronaviruses may require a second factor for their entry in specific host cells (Yokomori and Lai, 1992b). Surprisingly, a different molecule, human aminopeptidase N is the receptor for human coronavirus 229E, a virus which is related to the mouse MHV (Yeager et al., 1992). This cell surface glycoprotein, found in a synaptic fraction, is thought to help break down neurotransmitters in the brain and could allow human coronaviruses to enter cells in the nervous system (see below) (Murray et al., 1992; Stewart et al., 1992). Most recently, the membrane cofactor protein CD46 has been identified as a functional receptor for measles virus (Dorig et al., 1993). This protein is widely distributed on human cells and is a member of the regulators of complement activation gene cluster on chromosome 1.

HSV-1 can use as receptors heparan sulfate moieties of cell surface proteoglycans which are widely distributed on animal cells (Sieh et al., 1992). However, viral entry can still occur—although at low efficiency—in a mouse L cell mutant defective in the synthesis of these glycans (Gruenheid et al., 1993). Finally, HIV-1 uses the CD4 molecule, which functions in the recognition process between T cells and antigen presenting cells, to enter not only T cells and monocyte-macrophages but also microglial cells (Jordan et al., 1991). In some cells, an additional molecule may interact with a region of the HIV-1 surface glycoprotein (gp120) toward the amino terminus from the CD4 binding site called the V3 loop and involved in virus tropism (see below). Interestingly, galactocerebroside, a glycolipid highly expressed on oligodendrocytes, can serve as an alternate receptor for HIV-1 in central nervous system cell lines (Harouse et al., 1991). Thus, viruses use cell type-specific surface molecules, some of them with important functions, for their binding and entry.

Is There a Particular Time at Which Viral Infections of Neuroglia are Occurring?

With viruses that show tropism for both neurons and glia, virus expression tends to be confined to neuroglia when the organism becomes immunocompetent and the nervous system cells is differentiated. For instance, the JHM strain of mouse coronaviruses will cause acute lethal encephalitis in newborn rats and mice while it induces a demyelinating disease in older animals (see below) (Weiner et al., 1973; Sorensen et al., 1980). In addition, viral persistence is more likely to occur in the nervous system in the face of a more mature immune and nervous systems as observed in measles virus infection of rodents (reviewed in Dubois-Dalcq, 1979). On the other hand,

intrauterine infection may affect a specific neuroglial cell at a critical stage of development, hampering its differentiation. Such mechanisms may be at play in the hypomyelination observed in lambs affected with border disease, an infection caused by a sheep pestivirus (Potts et al., 1985). However, all types of central nervous system cells are susceptible to this virus *in vitro* (Elder et al., 1987). Infection of ependymal cells *in utero* is particularly frequent in congenital infections with RNA viruses, often resulting in hydrocephaly in animals (reviewed in Coyle, 1991). In humans, cytomegalovirus can infect ependymal cells *in utero* in the first half of pregnancy. Individual viral proteins may also have a detrimental effect on neuroglial cell development as suggested by experiments with transgenic mice, which express the early region and T antigen of the JC papovavirus in oligodendrocytes and develop dysmyelination (Small et al., 1986). However, it is not known whether viral proteins may act in this way in the course of natural infection.

What Factors Influence the Outcome of Viral Infections of Neuroglial Cells?

Viruses may cause cytopathic effects such as cell fusion (in microglia or astrocytes) or lysis (in oligodendrocytes) which can be followed by gliosis, formation of microglial nodules and/or demyelination. Of great influence on the outcome of these viral infections is the way the immune system acts within the central nervous system as discussed in Chapters 44, 45, and 46. Antibodies as well as interferon-regulated antiviral genes are important in limiting viral spread (Arnheiter et al., 1990). However, viral infections of neuroglia can only be efficiently cleared by cytotoxic T cells. As activated T cells are able to migrate in and out of the central nervous system (Hickey et al., 1989), cytotoxic *T* cells sensitized to viral antigens present in the central nervous system will interact with antigen-presenting cells such as microglial cells, which can express class I and II molecules (reviewed in Sedgwick and Dorries, 1991). When clearing of viral infection is complete, neuroglial cells are able to regenerate and remyelinate as seen in rats and mice. Depending on the genetic makeup of the MHC complex, the host susceptibility to viral infection and the establishment of viral persistence in the nervous system will vary (Brahic et al., 1991). If viral infection is prolonged, T cells may become sensitized to host antigens such as myelin proteins (myelin basic protein [MBP], proteolipid protein [PLP]), leading to autoimmune demyelination. Sensitization to MBP has been demonstrated in rats infected with JHM coronavirus (Watanabe et al., 1983) or with measles virus (Liebert et al., 1987). Besides, measles virus infection of the central nervous system enhances its susceptibility to autoimmune T cell aggression (Liebert et al., 1990).

Another possibility is that molecular mimicry between viral proteins and host nervous system proteins may allow autoimmunity to develop after removal of the initial immunogen ("hit and run" event; reviewed in Oldstone et al., 1987). For instance, a measles viral peptide corresponding to a sequence in this virus phosphoprotein is homologous to a 8-10 amino acid encephalitogenic site of rabbit MBP and can induce inflammatory cells accumulation around the vessels in the central nervous system. Such molecular mimicry and "hit and run" events may explain why cerebrospinal fluid lymphocytes proliferate in response to both viral and host antigens in acute postinfectious encephalomyelitis in which virus can rarely be isolated from the brain (Oldstone, 1987). Finally, virus infection microglia and macrophages may indirectly affect other nervous system cell types (Giulian et al., 1990) by triggering astrocytes to synthesize neurotoxic cytokines as suggested by *in vitro* experiments with HIV-1 (Genis et al., 1992). Besides viral proteins small metabolites released by activated microglia may damage neurons and/or oligodendrocytes or alter their specialized functions (see below).

Are There Viral Molecular Determinants That Control Tropism for a Particular Nervous System Cell Type, Neurovirulence, and Viral Persistence?

A striking example of control of tropism by viral determinants is the case of mouse reoviruses in which the type 1 specifically infects ependymal cells and causes hydrocephalus, while the type 3 only infects neurons and causes acute meningoencephalitis. As reoviruses have a multiple segmented genome, infection with viruses containing reassorted segments showed that the viral S1 gene encoding the viral outer capsid sigma 1 determines this cell tropism (for review see Tyler and Fields, 1990). Determinants of viral persistence have been studied in mouse picornaviruses and demyelinating diseases are often associated with reduced virulence and cytopathogenicity of the virus (see below). Moreover, when the virus resides in the brain for prolonged periods of time, it may evolve and undergo repeated mutations and/or deletions, resulting in the emergence of "brain-specific variants" with defects favoring persistence inside the infected cells in a form invisible to the immune system (lentiviruses, measles virus, JC

virus). Thus the viral genetic makeup and the evolutionary changes of neurotropic viruses in the brain are important determinants of disease pathogenesis.

I will now describe specific examples of viral infections of glia in animals and men. I have grouped the infections of astrocytes and oligodendrocytes together because viruses rarely infect only myelin-forming cells. Microglial infections will be dealt with in a separate section.

VIRAL INFECTIONS OF OLIGODENDROCYTES AND ASTROCYTES

Infections with Theiler Murine Encephalomyelitis Virus

The nonvirulent DA strain of the Theiler murine encephalomyelitis virus (TMEV); a picornavirus, causes a biphasic disease of the central nervous system in SJL/J mice inoculated intracerebrally at 4 to 5 weeks of age. In this TMEV model, infection of neurons appears to be a prerequisite of the infection of neuroglia. An initial phase of encephalitis and infection of the spinal cord recedes and is followed by a persistent infection of neuroglial cells and demyelination (Patick et al., 1990a) (Figure 67-1A). Scattered neuroglial cells expressing both viral RNA and proteins are found in animals persistently infected with TMEV (Figure 67-1B). Although oligodendrocytes are most frequently infected, astrocytes and microglia can also express viral RNA (Aubert et al., 1987). Deletion analysis indicates that determinants of persistence and demyelination are located between the C-terminal end of the VP3 capsid protein and the N-terminal extremity of protein 2A, as the VP1 gene can attenuate the highly virulent GD VII strain when chimeric viruses are constructed (Tangy et al., 1991, 1993). Other studies with intratypic recombinant viruses indicate that multiple gene segments influence the number and extent of demyelinating lesions (Rodriguez and Roos, 1992).

The susceptibility to this mouse virus-induced demyelinating disease is genetically determined (Patick et al., 1990b). Congenic mice with different MHC haplotypes indeed show either no lesions or different degrees of demyelination after inoculation with the DA strain. Resistance appears determined by efficient presentation of viral antigen to the immune system, resulting in local clearing of the virus and prevention of demyelination. Two different loci determine the persistence of the virus and demyelination: the H2D region of the MHC complex and another locus outside of this complex (Bureau et al., 1992). Thus it is likely that CD8+ cytotoxic T cells play a major role in the outcome of this central nervous system viral infection. To test this hypothesis, DA virus was inoculated intracerebrally into beta$_2$ microglobulin knockout mice, which fail to express class 1 MHC molecules, and therefore lack CD8+cells (Fiette et al., 1993). Although these beta$_2$ microglobulin-deficient mice were of a strain normally resistant to TMEV infection, they failed to clear virus and went on to develop demyelination. Thus CD8+ cells are required to clear the virus but do not appear to mediate demyelination, raising the possibility that viral persistence in oligodendrocytes may alter the expression of myelin genes.

Interestingly, certain treatments of DA-infected animals can decrease the extent of demyelination and can even enhance repair (Patick et al., 1991). For instance, intraperitoneal injection of tumor necrosis factor-alpha considerably decreased the degree of demyelination observed after intracerebral inoculation of the DA strain in SJL/J mice, even though virus expression is not affected (Paya et al., 1990). Furthermore, intraperitoneal injection of immunoglobulins from mice immunized against spinal cord homogenate caused enhanced remyelination in these infected mice (Rodriguez et al., 1991) (Figure 67-1C).

Infection with Mouse Coronaviruses

Certain strains of MHV replicate in the central nervous system; the JHM strain is particularly neurotropic for both rats and mice (Weiner et al., 1973; Sorensen et al., 1980). When inoculated intracerebrally at 3 to 4 weeks of age, the MHV-A59 strain replicated in some strains of mice (such as C57 BL/6 and BALB/c; SJL/J are resistant), causing a paralytic disease with demyelination at 2 to 4 weeks (Godfraind et al., 1989. Jordan et al., 1989). MHV-A59 can trigger a rigorous immune response which cleared the virus from the demyelinating lesions, allowing the nervous tissue to react to injury and repair myelin. Remyelination started around 4 weeks and continued for the following 8 to 12 weeks. *In situ* hybridization studies of myelin gene expression in the first week after viral infection showed a rapid loss of myelin gene transcripts concomitant with active viral replication in oligodendrocytes before demyelination was observed (Jordan et al., 1989). At 3 weeks, however, new myelin gene transcripts were already detected in the lesions, that is, before remyelination started (Jordan et al., 1990). The first transcripts to be detected were the early developmental forms of MBP mRNAs which were increased up to 17 times over control. It thus appears that

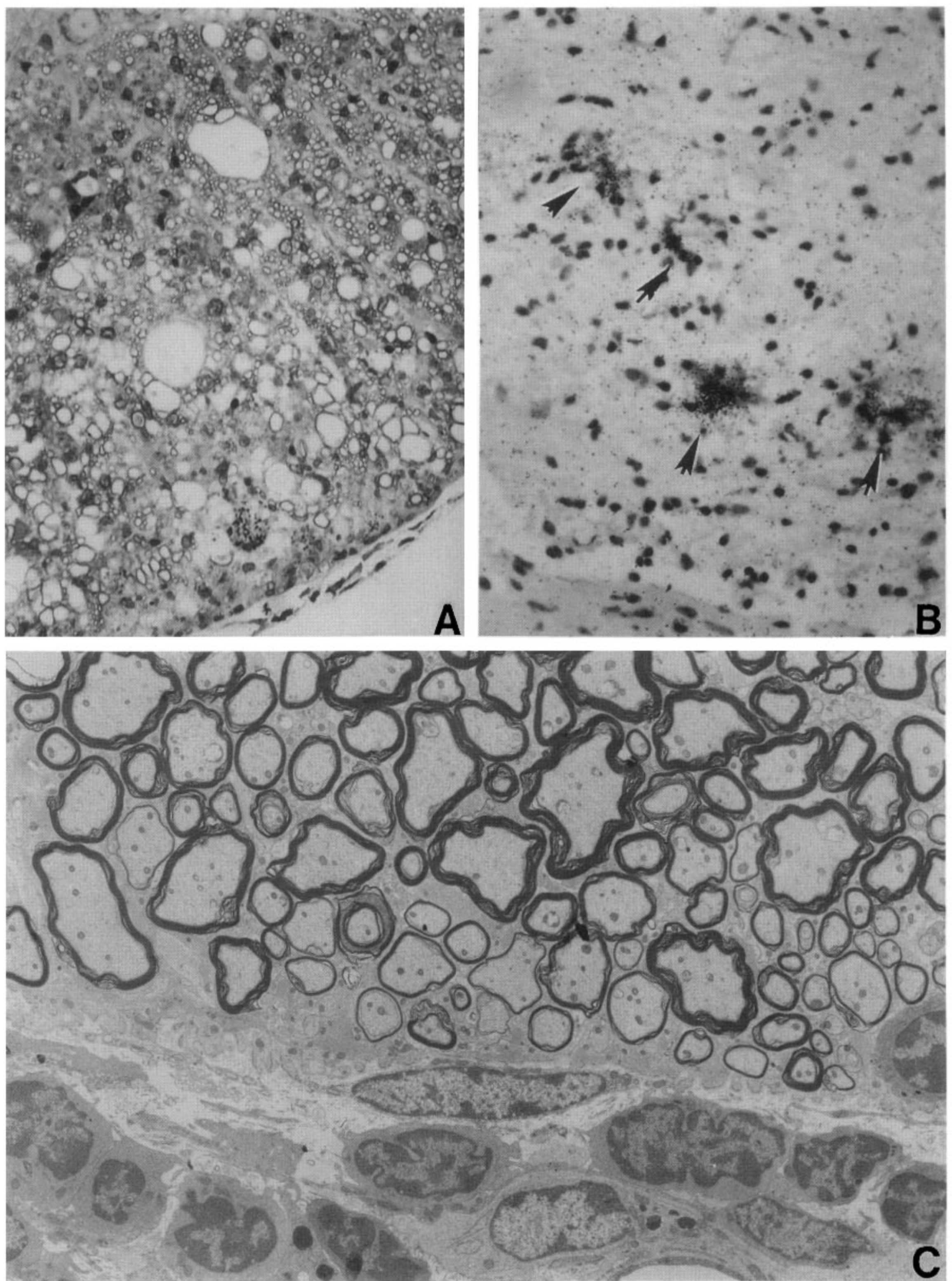

FIG. 67-1. Biological features of the demyelinating disease caused by the DA strain of TMEV in SJL/J mice. (**A**) Cross section through the spinal cord of mice infected for 45 days reveals extensive inflammation and prominent myelin destruction with preservation of the axons (glycol methacrylate embedding and erichrom/cresyl violet staining). (**B**) Combined immunocytochemistry and *in situ* hybridization for TMEV-specific RNA and protein reveals scattered positive cells in the spinal cord white matter of infected mice. (**C**) Mice infected for several months with TMEV were treated with spinal cord homogenate antiserum for a month: multiple remyelinated axons in the vicinity of an intense inflammatory response surrounding a capillary are seen in this electron micrograph (uranyl acetate and lead citrate; ×4900). [All micrographs are courtesy of Dr. C. Rodriguez with permission of the Society for General Microbiology. Figure A: Patick et al. (1990) and of the U.S. and Canadian Academy of Pathology; Figure C: Rodriguez et al. (1991).]

regenerating oligodendrocytes can reset their myelination program after viral infection has been cleared.

Oligodendrocyte precursors were found to proliferate throughout the spinal cord of A-59 infected mice already at 1 week postinoculation and up to 4 weeks (reviewed in Dubois-Dalcq and Armstrong, 1990). When cultured rat oligodendrocytes at different stages of development were inoculated with the JHM strain, few cells of the oligodendrocyte lineage became infected if they were maintained at a precursor stage by the addition of mitogens (Pasick and Dales, 1991). Thus progenitor cells are less susceptible to viral infection and may quickly respond to mitogenic signals in the lesion during the early phase of the disease (Godfraind et al., 1989). This may favor the birth of new oligodendrocytes with rapid remyelination and recovery in these rodents.

It is possible that human coronaviruses such as the 229E strain (which causes pulmonary infections) may occasionally invade the central nervous system as suggested by a recent reverse transcriptase-polymerase chain reaction analysis of RNA extracted from multiple sclerosis brain. Specific 229E sequences were detected in 4 of 11 patients but in none of 11 controls, including patients with other neurological diseases (Stewart et al., 1992). An other study has identified coronavirus mRNA by *in situ* hybridization in 12 of 22 multiple sclerosis brains (Murray et al., 1992).

Infections with Mouse Retroviruses Containing the Bacterial Galactosidase Enzyme Gene LacZ

Mouse retroviruses expressing the LacZ gene (Sanes et al., 1989) have been used to follow the fate of dividing neuroglial cell precursors in the developing brain. The BAG virus infected one precursor cell which migrated into the white matter, divided, and differentiated into myelinating oligodendrocytes. Four weeks after injection of the virus in the newborn rat subventricular zone, the white matter contained oligodendrocytes expressing LacZ in their entire cytoplasm and myelin internodes (Figure 67-2).

Infections with Paramyxoviruses

On rare occasions, measles virus can invade the central nervous system and persist in the human brain, causing two lethal diseases known as subacute sclerosing panencephalitis (SSPE), occurring 5 to 10 years after the initial measles virus infection and measles inclusion body encephalitis (MIBE) in immunocompromised hosts (for review see Schneider-Schaulies and Liebert, 1991). The virus can infect both neurons and neuroglial cells, including oligodendrocytes, where characteristic type A Cowdry inclusions made of viral nucleocapsids can be seen in the nucleus (for review see Dubois-Dalcq, 1979). Although this RNA virus replicates in the cytoplasm, uncoated measles virus nucleocapsids can indeed migrate to and accumulate in the nucleus. Experimental models of measles virus persistence in the central nervous system have been reviewed (Rammohan et al., 1983). In humans, it is not clear how and when the virus enters the central nervous system and why it stays silent for so long, causing SSPE only in rare cases. Measles virus strains persisting in the brain of SSPE patients were found to be defective as mutations accumulated in all viral genes, particularly the hemagglutinin (HA) and M genes (for review see Billeter and Cattaneo, 1991). For instance, 20 A to G mutations were found in the HA gene of an SSPE virus with strongly reduced hemadsorption, and 50% of the C residues were changed to U in the M gene of a patient with MIBE (Cattaneo et al., 1989; for review see Schneider-Schaulies and Liebert, 1991). This biased hypermutation is probably due to the action of a double-stranded RNA/unwinding/modifying enzyme acting on double-stranded regions between plus and minus measles virus RNA strands, which may form through faulty viral transcription or replication (ibidem). The M protein of a neurovirulent SSPE strain—which differed from its progenitor measles virus by 15 amino acid substitutions—failed to associate with the nucleocapsid and stayed diffuse in the cytosol instead of associating with the plasma membrane, a step necessary for viral budding (Hirano et al., 1992).

There are several other factors that play a role in measles virus persistence in the central nervous system (for review see Schneider-Schaulies and Liebert, 1991). Central nervous system cells can downregulate measles virus transcription more than other cell types. Consequently, little viral envelope genes are expressed and infected cells escape immune recognition, allowing the infection to perpetuate in the face of a strong immune response. Specific cytotoxic T cells recognizing the measles virus nucleocapsid protein could, however, clear the infection if antigens were presented in the context of class 1 molecules, but these are usually not expressed by neurons and oligodendrocytes in which the virus resides. Thus, in SSPE, the infection persists unabated.

Canine distemper virus (CDV), closely related to measles virus, causes a demyelinating disease in dogs known as Carre disease. Both astrocytes and macrophages in spinal cord lesions have been shown to contain viral inclusions made of nucleocapsids (for

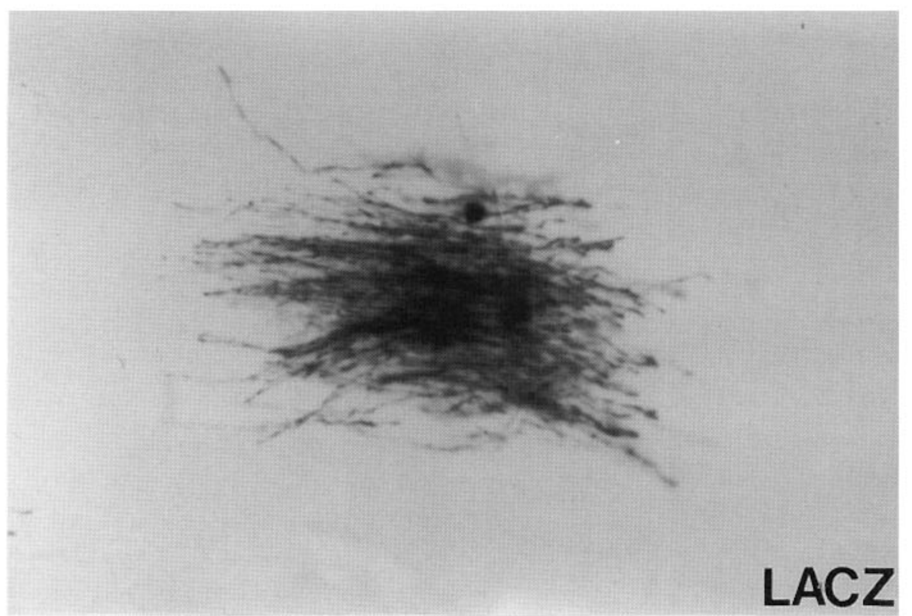

FIG. 67-2. Four oligodendrocytes and their numerous myelin sheaths all highly express the *E. coli* LacZ gene 4 weeks after injection in the subventricular zone of a recombinant retrovirus containing this gene (BAG retrovirus). The virus infected one precursor cell that migrated into the white matter, divided and differentiated into myelinating oligodendrocytes. The blue reaction product of the enzyme—XGal is diffuse throughout the cell body and processes, including the myelin internodes. [Micrograph courtesy of S. W. Levison and J. E. Goldman at Columbia Medical school, New York.]

review see Dubois-Dalcq et al., 1979). Antimyelin antibodies have been found as well as images of stripping of myelin by mononucleated cells, suggesting an autoimmune component to this chronic viral demyelinating disease.

Infections with Human JC Polyomavirus

JCV specifically infects human oligodendrocytes in immunocompromised hosts, causing a disease called progressive multifocal leukoencephalopathy (PMI). The virus accumulates in the nucleus of oligodendrocytes (Figure 67-3A, B) and causes lysis, leading to the formation of small foci of demyelination which then fuse and form extensive lesions (for review see Major et al., 1992). In contrast, astrocytes —often enlarged and called "bizarre"—also harbor viral DNA but rarely harbor virions or show lysis *in vivo*. Cultured astrocytes, however, can support JCV replication and assembly of virus. *In vitro*, the virus grows best in human fetal neuroglial cells and astrocytes as well as Schwann cells, but not in neurons. When inoculated in hamsters and owl monkeys, JCV caused astrocytomas and medulloblastomas where the JC T antigen was sometimes detected prior to tumor formation (Ressetar et al., 1990). There is, however, no known association between human glioblastomas and JC virus expression.

JCV is a very ubiquitous virus; 65% of adolescents and 92% of adults in urban areas have JCV antibodies (reviewed in Major et al., 1992). The incidence of PML has increased with the occurrence of the AIDS epidemic (close to 4% of AIDS cases develop this disease). Virus DNA was detected in B cells in the bone marrow and in brain lesions (Hough et al., 1988). JCV genome has been detected by PCR in circulating white cells of PML patients and of HIV-1 positive patients without clinical symptoms of PML (Tornatore et al., 1992). However, JCV genomic sequences were not found in control and multiple sclerosis brains (Buckle et al., 1992), throwing doubt on the suggestion that JCV DNA may reside in non-PML affected brains (White et al., 1992). Thus, in some cases, this ubiquitous virus may spread to the brain and persists at this site without causing a demyelinating disease unless the immune system becomes compromised. Studies of the molecular makeup of JCV isolated from different patients and organs suggest the existence of an *archetype virus*—in kidney and urine of transplanted patients and pregnant women—and a *brain form*, which shows duplications and/or deletions in the noncoding regulatory region (discussed in Major et al., 1992; White et al., 1992).

Why does JCV infect specifically oligodendrocytes and astrocytes in the central nervous system? Class 1 MHC proteins are an essential component of the receptor for simian virus 40, which is closely related to JCV (Breau et al., 1992). The possibility must be considered that class 1 molecules, which are highly expressed in astrocytes and oligodendrocytes in PML lesions (Achim and Wiley, 1992), may serve as the JCV receptor. Moreover, host cell factors clearly play an important role in the restricted expression of JCV. In human fetal neuroglial cells, two proteins bind specifically to the 98 bp repeats present in the regulatory region of the MAD-1 strain of JCV, a prototype brain isolate (reviewed in Major et al., 1992).

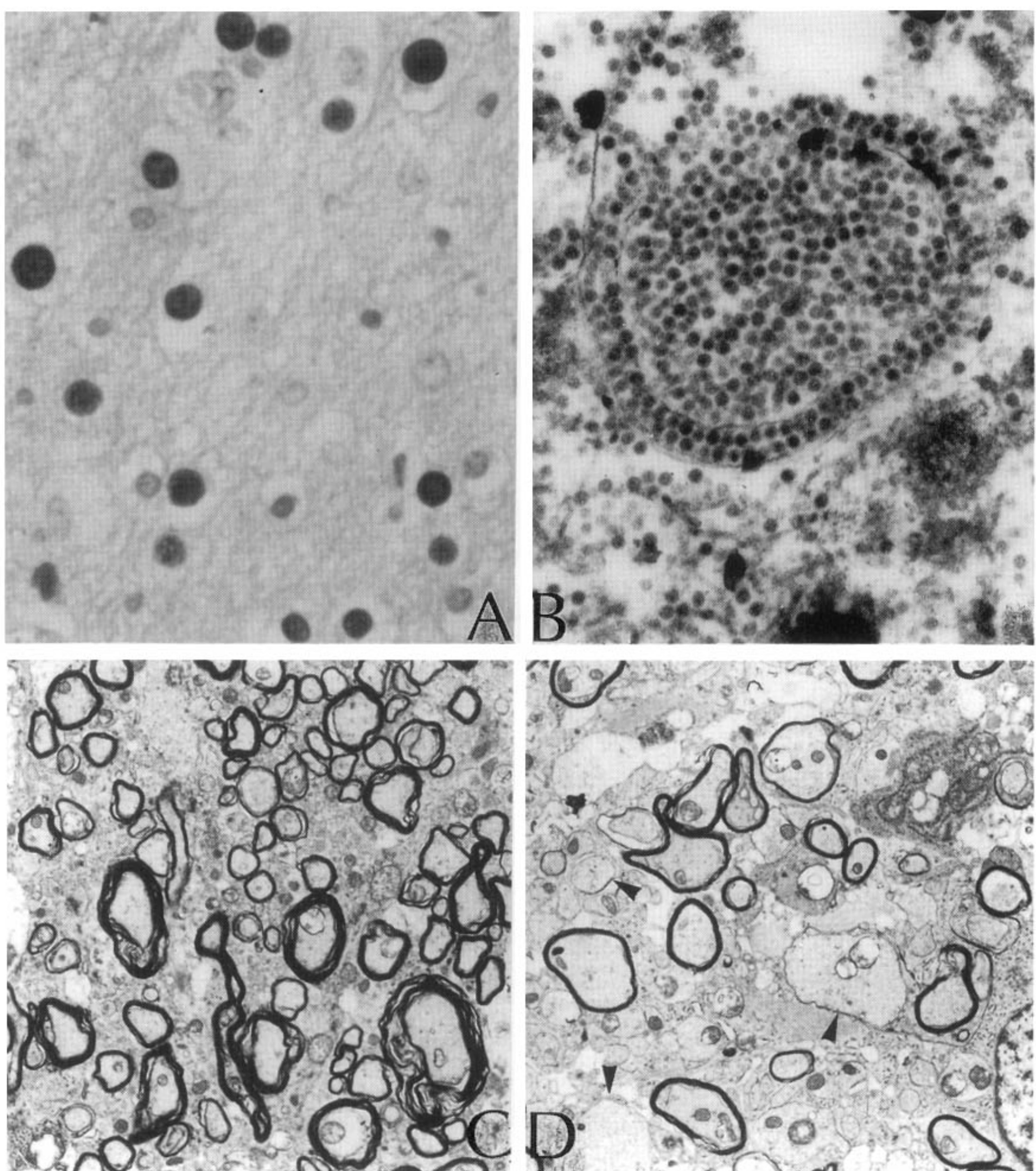

FIG. 67-3. JC virus infection and expression alters oligodendrocytes in the human disease PML and in *transgenic mice*. (*A*) JCV viral DNA was detected with a biotin-labeled DNA probe and immunoperoxidase histochemistry in the nuclei of most oligodendrocytes in a PML lesion. (B) Electron micrograph of the nucleus of an infected oligodendrocyte filled with papovavirions. (C and D) show the spinal cord of a control (*C*) and a transgenic mouse (*D*) expressing the SV40 large T antigen under the control of the JC promoter. The JC promoter drives the expression of the SV40 T antigen in oligodendrocytes and results in central nervous system hypomyelination (*D*). Figures A and B: courtesy of Major et al. (1992); and Berger et al. (1992). Figures C and D: courtesy of Feigenbaum et al. (1992), with permission of the American Society for Microbiology.]

Studies in transgenic mice have shown expression of the JCV early region and T antigen in oligodendrocytes *in vivo* (Small et al., 1986). The JCT antigen blocked expression of myelin proteins by oligodendrocytes, although some myelin gene transcripts were detected (Trapp et al., 1987). Tissue and cell specificity of the JC promoter was demonstrated in transgenic mice by exchanging the SV40 and JC regulatory regions to produce chimeric constructs designated JC (SV40) and SV40 (JC) (Feigenbaum et al.,

1992). The JC (SV40) constructs produced dysmyelination in transgenic mice (Figure 67-3C, D); in addition, choroid plexus tumors arose in these mice as they did in the SV40 (SV40) transgenics. In contrast, the SV40 (JC) caused choroid plexus tumors but no dysmyelination. The JC regulatory regions therefore play a crucial role in directing expression of T antigen in oligodendrocytes, resulting in myelin alteration.

Infections with Herpes Viruses in Human and Animal Models

HSV type 1 and type 2 are well known for causing latent infections of sensory ganglia, but they can also induce central nervous system demyelination in rodents. Mice inoculated with HSV-1 in the cornea developed infection in the trigeminal nerve at the root entry where astrocytes and oligodendrocytes produced virions and central nervous system demyelination was observed (Townsend et al., 1981). Intracerebral inoculation with HSV-2 is often lethal for mice, but the surviving animals developed demyelination in spinal cord and optic nerve (Martin, 1982). Viral antigen was found in axonal tracts showing demyelination, suggesting that virus could spread from the axon to several myelin internodes and their respective oligodendrocytes (for review see Martin et al., 1988) (Figure 67-5). It was therefore proposed that HSV-2 may have a demyelinating potential in humans when reactivated, but convincing evidence for this is still lacking.

Herpes viruses can notably infect many nervous system cells *in vitro*, among them Schwann cells. Varicella zoster virus (VZV) is no exception to this rule as it can infect cultured fetal human fetal Schwann cells and astrocytes (Assouline et al., 1990). After an initial disseminated infection in childhood, VZV becomes latent in DRG and may get reactivated years later, producing shingles. The possibility that the virus spreads not only in neurons but also in Schwann cells during the latency period has been raised. Another important pathogen in humans, cytomegalovirus, infects white cells and cells of the monocyte-macrophage lineage, which can invade the peripheral nervous system roots and dorsal root ganglia, as seen in patients with AIDS (Behar et al., 1987). The virus can also invade the brain where it infects the ependyma lining the ventricles and forms characteristic "cytomegalic cells," which contain viral inclusions and are most likely cells of the monocyte-macrophage lineage. In AIDS patients, cells containing both cytomegalovirus and HIV-1 proteins have been detected in ventriculoencephalitis cases (Fiala et al., 1992).

VIRAL INFECTIONS OF MICROGLIA AND BRAIN MACROPHAGES

Microglia and brain macrophage infections are mostly caused by retroviruses that show dual tropism for certain white blood cells and the central nervous system. (See Chapter 44 for details on the normal functions of microglia.)

Infections with Neurovirulent Mouse Ecotropic Retrovirus CasBrE

The Lake Casitas wild-mouse population has a high incidence of infectious murine leukemia virus infection, and these wild mice can develop two different diseases, a pre-B cell lymphoma (18%) and a hindlimb paralysis (12%) (for review see Portis, 1990). As determinants of neurovirulence reside in the *Env* gene, a chimeric virus was constructed with *Env* gene inserted into Friend MuLV (FrCase) (Lynch et al., 1991). This highly neurotropic virus caused neuronal spongiform degeneration, as seen in the wild-mouse disease, with postsynaptic vacuolation in the absence of gliosis (Lynch et al., 1991). However, viral RNA and proteins were not detected in these altered neurons but were present in microglial cells, which produced viral particles (Figure 67-4 A, B, C). Viral replication could also occur in endothelial cells and pericytes throughout the central nervous system. In contrast to the spinal cord neurons, granular neurons of the cerebellum were supporting viral replication without any signs of cytopathology. These observations suggest that the neurodegenerative disease is an indirect consequence of viral infection, as may also be the case with other retroviral infections of the nervous system (see below).

Infection with Lentiviruses (Visna, HIV-1, SIV)

Visna Virus Infection of Sheep. Natural visna virus infection of the sheep has long been proposed as model for human demyelinating diseases (for review see Nathanson et al., 1985; Zink et al., 1991). Both visna virus and HIV-1 are related members of the family of lentiviruses and can infect the brain. HIV-1 infects both T cells and macrophages, resulting in immunodeficiency, while visna virus infects primarily differentiated macrophages. Visna virus is probably transported to the brain in infected monocytes, and viral replication is triggered when the infected cells differentiate into macrophages. Infected sheep usually show repeated episodes of central nervous system inflammation and demyelination, followed by myelin repair, with numerous CD8 cytotoxic/suppressor T cells present in the perivascular cuffs. Double-labeling for macrophage markers and viral mRNA have con-

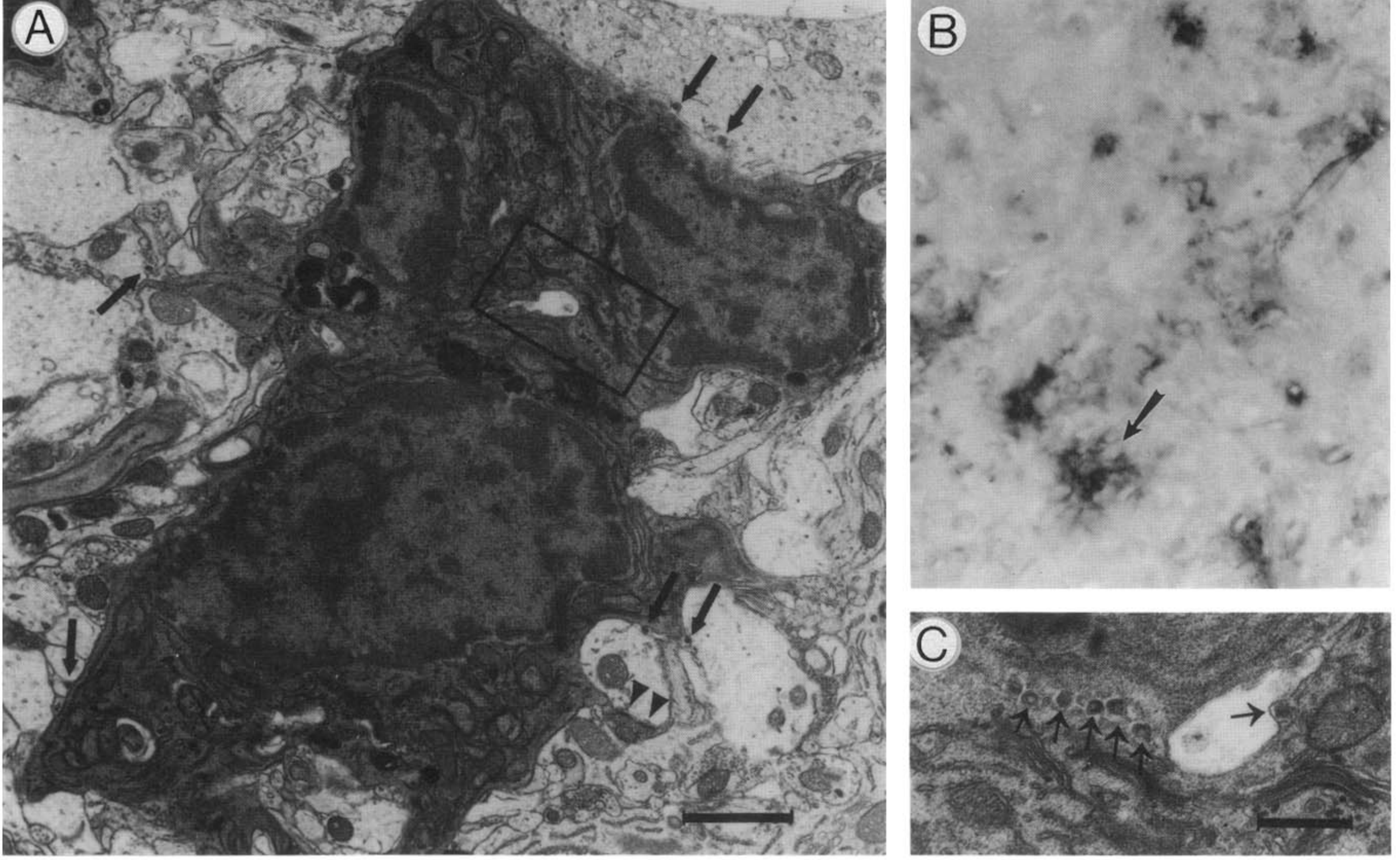

FIG. 67-4. Infection of microglial cells in mice infected with a highly neurovirulent recombinant form of the wild mouse ecotropic retrovirus (FrCasE). (**A**) Electron micrograph of an infected microglial cell in the lateral vestibular nucleus 17 days postinoculation. Note the dark cytoplasm, lipofuscin granules, and ropelike endoplasmic reticulum typical of ramified microglia. Budding viruses are seen between microglia and the adjacent surrounding vacuoles in the box enlarged in **C**. (**B**) Env protein staining in scattered ramified microglial cells (*arrows*) as well around the vessels. Bar = 1 μm. [Courtesy of Lynch et al. (1991), with permission of the Cell Press.]

firmed that macrophages and/or microglia are the predominant host cells for viral replication in the brain (Zink et al., 1991). Moreover, these infected cells, when activated by cytokines such as gamma-interferon, produce more virus and express MHC class 2 antigens. In addition, some other neuroglial cells may be latently infected, harboring proviral DNA detectable in infected tissues by highly sensitive methods such as *in situ* polymerase chain reaction amplification (Staskus et al., 1991).

Infections with HIV-1 and Related Simian Viruses. HIV-1 causes the AIDS-associated psychomotor complex in a substantial proportion of patients, who develop either neuropathy, encephalitis, leukoencephalopathy, myelopathy, or diffuse gray matter poliodystrophy (Budka et al., 1991). The neuropathological lesions range from gliosis to demyelination, and sometimes neuronal loss (for review see Dubois-Dalcq et al., 1990; Spencer and Price, 1992). SIV also causes a very similar meningoencephalitis (Desrosiers et al., 1991). There is overwhelming evidence that the major target of HIV-1 infection in the brain are cells of the monocyte-macrophage lineage, including microglia. Scattered microglial cells often express viral protein *in vivo* (Dickson et al., 1993) (Figure 69-5) and *in vitro* where cultured human microglia produce virions from their cell surface (Figure 67-6 C). HIV-1 strains with tropism for monocyte-macrophage (including the brain isolate JrFL) replicated well in primary human brain cultures, while none of the T cell adapted isolates grew in these cultures (Watkins et al., 1990). HIV-1 caused a progressive clustering and fusion of infected microglial cells (Figures 67-6A and B) mimicking the two pathological hallmarks of HIV-1 infection in the central nervous system: microglial nodules and multinucleated giant cells. Differentiated cells of neuroectodermal origin could not be infected by either a lymphotropic virus (LAV or LAI) or the monocyte/macrophage tropic strain JrFL (Sharpless et al., 1992b). Neither primary astrocytes from adult human brain nor differentiated neuronal cells (HCN-1) allowed the virus to go through cycles of reverse transcription and replication. Similarly, human oligodendrocytes did not get productively infected by these viral strains and developed a normal phenotype in the presence of ongoing infection of microglial cells. Thus, HIV-1 entry and/or expression in differentiated central nervous system cells appears to be a rare event or inefficient process. The situation may be different in the less mature brain as rare astrocytes were found to express HIV-1 in some cases of pediatric AIDS (Tornatore et al., 1994).

It appears that the neuropathological alterations in AIDS may be indirectly caused by viral and/or excitotoxic factors or cytokines released by infected microglia and macrophages in the brain (for review see Dubois-Dalcq et al., 1991). Moreover, specific interaction between infected monocytes/macrophages and astrocytes may trigger the production of neuronotoxic factors such as tumor necrosis factor-alpha, interleukin 1-beta, and arachidonic acid metabolites (Genis et al., 1992; Tyor et al., 1992; Dickson et al., 1993). It is interesting that the HIV-1 viral glycoprotein gp41 shares a common epitope with an astrocyte protein and that antiastrocyte antibodies recognizing this epitope are found in the cerebrospinal fluid of

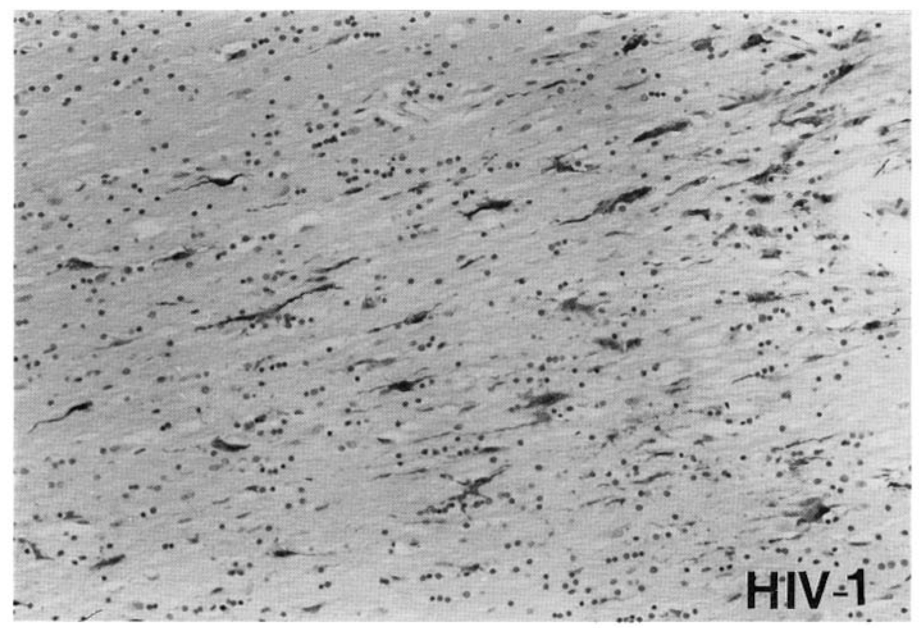

FIG. 67-5. Microglial cell infection in the AIDS encephalitis as demonstrated by immunostaining for gp 41 viral glycoprotein in the internal capsule of an AIDS patient. (Adjacent section stained with hematoxylin and eosin showed no obvious pathology.) [Courtesy of D. W. Dickson at Albert Einstein College of Medicine, New York.]

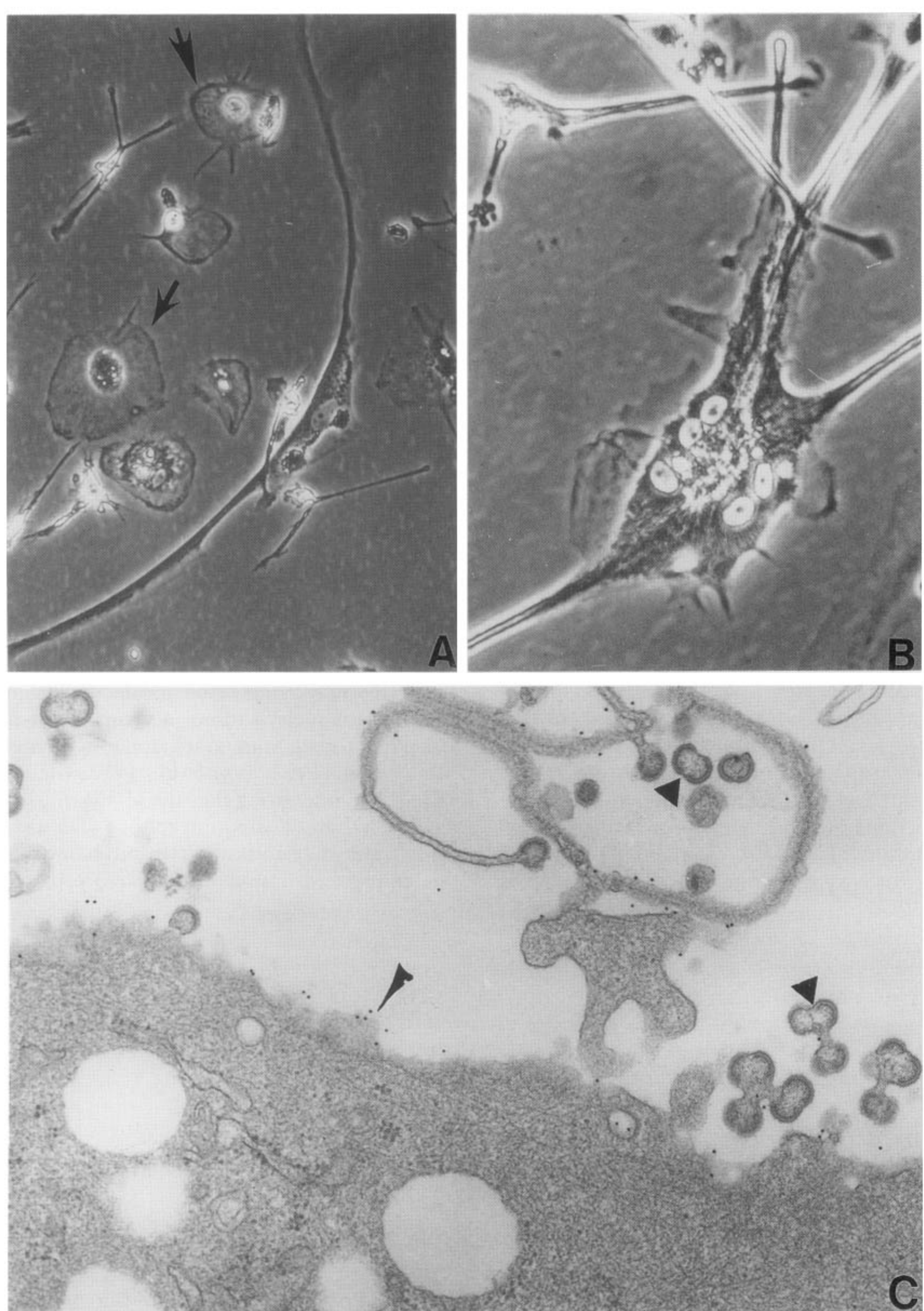

FIG. 67-6. Infection of cultured human microglia with HIV-1 strain AD-87 or ADA (Watkins et al., 1990; Genis et al., 1992). (**A**) Uninfected cultures contain ameboid (*arrows*) and elongated microglial cells, (**B**) At 12 days postinoculation with the virus giant multinucleated cells are prominent. When such cells are labeled in the cold with immunogold for the Leu M5 antigen characteristic of cells of the monocyte-macrophage, typical HIV-1 lentiviral particles are seen budding from the membrane of cells bearing gold particles. ×51,000.

AIDS patients with neurological complications (Yamada et al., 1991) (see discussion above on molecular mimicry).

The HIV-1 promoter—called the long terminal repeats or LTR—can direct the expression of the LacZ gene in transgenic mice (Corboy et al., 1992). Mice generated with central nervous system-derived LTRs (from the JrFL and JrCSF strains derived from the same patient) directed expression of LacZ to the nervous system, particularly in neurons, but also in some microglial cells and astrocytes. As HIV-1 LTRs can drive gene expression in a variety of transfected cell types *in vitro*, these transgenics observations may reflect the lack of host cell constrains on expression of the HIV-1 genome, except in situations where specific LTR mutations occur. The lack of evidence of infection of neurons by HIV-1 in the natural disease suggests that the restricted expression of HIV-1 in the brain of infected patients may be mostly due to constraints on viral entry rather than cell-specific intracellular factors.

Does the CD4 receptor mediate HIV-1 entry in the central nervous system, and are there specific molecular determinants in the virus that control tropism for central nervous system-derived microglial cells? Using RT/PCR on enriched microglial cells populations, CD4 transcripts were detected in populations enriched in microglia (Jordan et al., 1991). Viral infections of human brain cultures with monocyte/macrophage tropic strains can be prevented by preincubation with antibodies recognizing the CD4 binding site for the gp 120 envelope protein of HIV-1. To determine whether the *Env* gene of HIV-1 plays a role in microglial cell tropism, chimeric viruses were tested, which contained either the entire *Env* gene or portions of this gene from the JrFL neurotropic strain inserted into a lymphotropic strain genome (Sharpless et al., 1992a). These experiments showed that a region of the gp120 glycoprotein encompassing the V3 immunodominant loop was essential for efficient entry in microglia and that the same molecular determinants controlled microglial cell tropism and monocyte/macrophage tropism. Interestingly, an antipeptide antibody specific for the V3 loop of JrFL brain isolate could block viral entry in human brain microglia. Therefore, both the CD4 receptor and the V3 loop are potential targets for therapeutic strategies aiming at blocking HIV-1 spreading to the brain.

Infections with Human T Cell Leukemia Virus (Oncovirinae)

HTLV-1-associated myelopathy or tropical spastic paraparesis (HAM/TSP) is a chronic neurological illness occurring in clusters in various parts of the world (Japan and the Caribbean Islands; some areas of Africa, South America, and Indonesia), and the virus spreads by heterosexual contacts and/or transfusion (for review see Gessain and Gout, 1992). The neuropathology consists of chronic meningomyelitis with perivascular cuffing made mostly of CD8+ cells, demyelination with axonal loss, and discrete gliosis. It is unclear why the same virus can cause paralysis in some patients and acute T cell leukemia in others. TSP patients have elevated HTLV-1 antibodies and viral-specific cytotoxic T cells. Circulating white cells contain many enlarged activated T cells—some are called "flower cells"—expressing MHC class 1, showing increased proliferation *in vitro* and often carrying the viral genome (50% CD4 cells) and proteins. (These cells are not monoclonal or transformed as in acute T cell leukemia.) Cytotoxic T cells, recognizing the regulatory Tax protein, have been found in the cerebrospinal fluid and may target nervous system cells expressing the virus. Which cell type is infected in the central nervous system of TSP patients is unclear, but the virus can infect cultured monocyte/macrophages (Koralnik et al., 1992) and microglial cells with increased expression of interleukin-6 and tumor necrosis factor-alpha by these HTLV-1 infected cells (Hoffman et al., 1992). In inoculations of primary human brain cultures with two different strains of HTLV-1, (one from an acute T cell leukemia patient and the other from a TSP case), a slow protracted infection with positive detection of the viral genome in the cultured brain cells was observed (I. Koralnik et al., unpublished observations). To determine whether the virus is latent or persistent in the central nervous system tissues of TSP patients and in which cell it resides, *in situ* PCR is now being used to detect single proviral DNA copies or a small number of RNA copies coding for specific viral genes. Another possibility is that, in TSP patients, infected T cells which have migrated to the spinal cord and/or brain are the only infected cells in the central nervous system and that these cells may recognize a central nervous system specific antigen, leading to autoimmune demyelination and axonal damage.

Intriguing is the observation that the expression of the HTLV-1 Tax gene under its own LTR in transgenic mice causes peripheral nerve tumors reminiscent of schwannomas (Nerenberg et al., 1987). This animal model is reminiscent of Von Recklinghausen's disease, but there is no evidence of involvement of HTLV-1 in this human affection. This raises the question of the relevance of such transgenic models to the pathogenesis of viral diseases. In transgenics, the viral genes are inserted in every cell of the body,

and viral gene expression can start early in development. In contrast, viruses come at a precise time in life and invade their host as intact infectious organisms, requiring stringent conditions for their entry, spreading, and replication, triggering a complex series of reactions of the immune system and nervous tissues. All these events then result in a particular viral-induced nervous system disease of which the molecular pathogenesis has to be studied with full awareness of the natural history of the disease. Nevertheless, transgenics may be useful models, particularly to study genes coding for proteins influencing the spreading and outcome of viral infection, such as viral receptors (Ren and Raccaniello, 1992) and antiviral intracellular factors (Arnheiter et al., 1990).

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68 Alzheimer's disease

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ALZHEIMER'S DISEASE: A CONTROVERSY FROM THE BEGINNING

The story of the Alzheimer's disease syndrome is a history of discovery and rediscovery. An animated discussion about its true nature started with its first description and has centered around one fundamental question ever since: What distinguishes Alzheimer's disease from normally occurring processes of aging? Based on Alzheimer's histopathological description, this chapter is largely descriptive. It is confined to the discussion of some fundamental glial reactions that accompany neuronal reactions and might thus be helpful in understanding the cellular pathophysiology of Alzheimer's disease (for review of the molecular biology of Alzheimer's disease see St. George-Hyslop, 1989; Beyreuther and Masters, 1991).

In 1906, Alois Alzheimer published a report of a peculiar disease of the cerebral cortex ("Über eine eigenartige Erkrankung der Hirnrinde"; A. Alzheimer, 1906). In the course of 4½ years, a 51-year-old woman became increasingly helpless, with severe signs of cognitive dysfunction, including defects in memory, attention, and planning, followed by failure of language, visuospatial functions, and a deterioration of general intelligence. Despite the resulting dementia syndrome, the patient's personality initially remained comparatively well preserved. Upon neuropathological examination the brain showed generalized atrophy with no further macroscopical changes. Using the silver-staining technique of his colleague M. Bielschowsky, Alzheimer found peculiar *neurofibrillary tangles* in areas where neurons had obviously been lost. In addition, the brain contained numerous small *miliare Herde* (miliary foci), later called *senile plaques* (Simchowicz, 1910). They were composed of an unusual and strongly birefringent material, namely, amyloid. These lesions were surrounded by fibrous glial cells (astrocytes) and a large number of cells loaded with lipids (microglia/macrophages) (Figure 68-1).

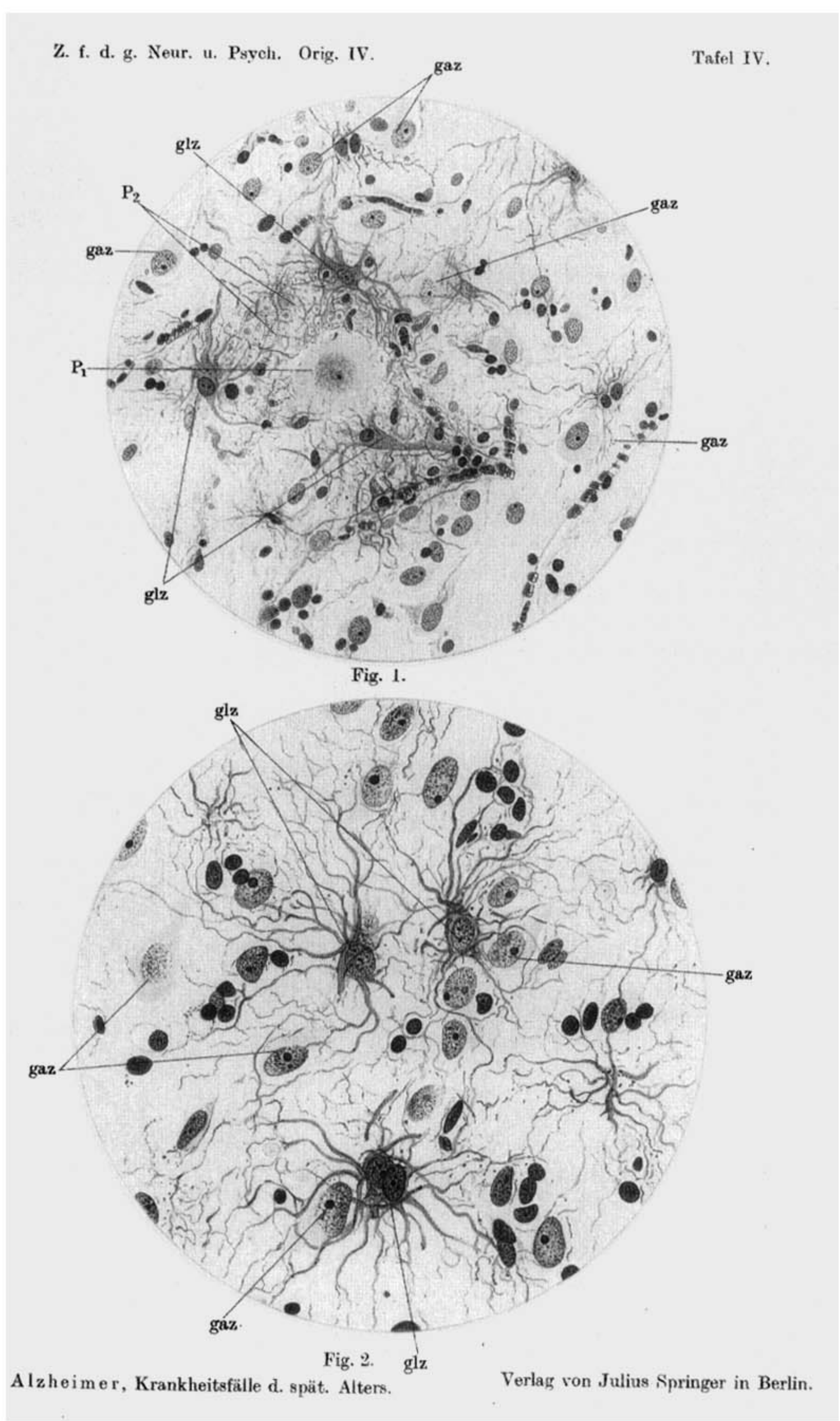
The first case of Alzheimer's disease ever to be described was sent from Frankfurt to A. Alzheimer, who was then assistant at the Psychiatric University Clinic in Munich. In 1911, after the diagnosis of the disease had been established, Alzheimer described a case of this disease in the 56-year-old day-laborer Johann F. The following is an excerpt from his detailed description, including most of the neuropathological features we know today (Alzheimer, 1911).

[General Appearance of a Brain with Presenile Dementia and Distribution of the Plaques]*

The microscopical investigation showed the cortex to be filled with . . . plaques to various degrees. The number of plaques generally corresponded with the macroscopically visible atrophy. In the frontal cortex they were found frequently, in the central gyri occassionally, in the parietal and partly in the temporal lobes in enormous masses, and in the occipital lobes, again, less frequently. No considerable differences existed between left and right. They were also frequent in the striatum, the nucleus lentiformis, and the thalamus. In the cerebellum they accumulated in individual lobuli, whereas in wide areas they were missing completely. Also in the grey matter of the pons, in different nuclei of the medulla oblongata, they were occassionally detectable. In the spinal cord, I saw only a single small plaque in the dorsal horn of a thoracic section.

Amongst the plaques of the cortex were quite a number of such extraordinary size as I have never seen them before, not even in the cornu ammonis of the senile dementia. They often reached through several layers. Some of them obviously resulted from fusion of smaller ones, because they harboured several central inclusions; others, however, showed a particularly large central body with an extremely wide "Hof" [halo of surrounding tissue]. Almost regularly, one could observe at the height of a lobe fewer plaques than at its sides and particularly at the base of a lobe. Also, in regions where they appeared particularly numerous, the plaques lay rarely in the first layer of the cortex, but rather started where the glial reticulum of the transition zone into the first small cellular layer fairly suddenly becomes denser. Occassionally, some very small plaques lay close to the border [of the transition zone]. They accumulated most prominently in the second and third layer; in the deeper

* For the sake of accuracy, faithfulness of translation has been given preference over a more fluent style, thus reflecting the tone of the original German text.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Alzheimer, Krankheitsfälle d. spät. Alters.
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cortex they became rarer, but in the white matter they were still numerous.

[Description of the Plaques and the Amyloid Deposits]

... Looking into a section of a particularly severely affected area fixed by Weigert's glial impregnation and stained with my modification of Mann's stain, we realize at low magnification dark spots of roundish, egg-shaped, often irregular form and different size, in extraordinary numbers. ... In the center of a great many foci, a large central body can be seen. It stands out due to its intensive blue staining. In smaller plaques, it [the central body] usually appears round, but at higher magnification not quite regularly delineated: in larger plaques its shape is more irregular; often one cannot see sharp borders, but some ragged protrusions disappearing in the surrounding, or it seems as if the central core disseminates into delicate, radially arranged spines. Otherwise, the core of the plaque appears homogeneous; cellular remnants cannot be seen.

... This core of the plaque in the cortex is regularly surrounded by a "Hof" [halo], which is brighter than the core but darker than the neighbouring tissue and usually several times bigger than the diameter of the core. Unlike the core, it [the "Hof"] is of a very complicated structure. First, we see slightly or more darkly stained, apparently homogeneous lumpy formations of very different size: from all their appearance, they are amorphous masses deposited here. Further, in the halo cellular elements can be detected, which, beyond doubt, have to be interpreted as glial cells. At one time large, at another time small, they sometimes show apart from their nucleus only little, slightly stained [Alzheimer used a modified Weigert's and Mann's staining procedure] [cyto-] plasm; again, at other times they are filled with greenish-yellowish substance with their nucleus displaced to the periphery, like we see this in "Körnchenzellen" ["granular cells", a term usually taken to describe activated microglia/brain macrophages] of any origin. Less frequently one sees forms of glial cells, which due to their small nucleus and evenly stained lobulated cell body one would like to count as ameboid [glia]. The proper fiber-forming glial cells do usually not lie within the halo of a plaque. However, in contrast to ordinary cases of senile dementia, we observe many more fiber-forming glial cells [astrocytes] which lie in the immediate vicinity of the plaque and send fibres into the halo but do not reach the core. They extend between the lumpy formations mentioned above. In rare cases, one observes how a fiber-forming glial cell ... sends into the halo an additional protoplasmic protrusion with a thick plump ending filled with green-coloured lipoid granules. Also "Stäbchenzellen" -like elements ["rod-cells", a term often used for microglia], obviously of glial origin, sometimes are lying in the halo; near both poles of the core is a satchel filled with greenish granules.

...

While in the cortex we hardly ever find in the core of a plaque without a halo, we come across many such halos without a core. ... However, in the cortex we find small and often irregularly shaped darker spots without clear inclusions so frequently that I must think that the just described fully differentiated plaques are preceded by a stage in which "[sich das] Gewebe verdichtet" [the tissue condenses]. ...

A particularly close relationship of the foci with the [blood] vessels cannot be observed .... it rather looks as if they [the foci] push aside the capillaries ...

Apart from the already mentioned pathological glial cell forms in the vicinity of the plaque, we generally see in the tissue, other than small, dark glial nuclei with little differentiated chromatin and very little [cyto-] plasm, accumulations of glial cells with a larger often oval nucleus and star-shaped cell bodies often covered with delicate dark dots. Regularly, light-refracting granules without clear staining are deposited within them in larger accumulations.

...

Fairly remarkable is the observation that in the numerous tissue sections from many areas of the brain not a single cell with the peculiar fibrillary degeneration [neurofibrillary tangles] I had described [earlier] could be found.

The impressive clinical picture, the early age of onset and the characteristic neuropathological findings led Emil Kraepelin, pioneer psychiatrist and Alzheimer's teacher, to postulate a new disease entity, which he called "Alzheimer's disease." Only a few years later, however, it was recognized that the plaques and tangles thought to constitute a specific sign of Alzheimer's disease were also frequently found in brains of nondemented old people (Gellerstedt, 1932; Braunmühl, 1957). This made Alzheimer eventually conclude: that "*... the plaques are not the cause of senile dementia but only accompany the senile involution of the central nervous system ...*" (Alzheimer, 1911). Recent studies confirm that the presence of plaques and tangles correlates imperfectly with the development of the dementia syndrome. It is the actual quantitative loss of synapses that seems more closely related to the severity of cognitive dysfunction (DeKosky and Scheff, 1990).

FIG. 68-1. Original drawing from A. Alzheimer (1911). Frozen section with glial staining. Weigert's glial stain, Zeiss 1/13. Fig. 1 drawn after tubus 140, Fig. 2 after tubus 160, compensation ocular 4. gaz. ganglion cell, glz. glial cell, P central part (core) of the plaque, P_2 peripheral part, "Hof" [halo] of the plaque.

Fig. 1. Relationship of the fiber forming glial cell with the plaque. Right upper parietal lobe. In the core of the plaque [is] a very small, obviously darkly iodine-stained central part surrounded by a darker and than a brighter ring. The peripheral part is penetrated by numerous extraordinarily delicate glial fibres, which stem from the glial cells lying at the fringe of the halo.

Fig. 2. Huge fiber forming glial cells from deeper layers of the cortex of the right upper parietal lobe often embracing neighbouring ganglion cells.

Today, according to the U.S. Department of Health and Human Services, an estimated four million Americans suffer from Alzheimer's disease. This large number includes all those patients who develop dementia at a later age, that is, *senile dementia of the Alzheimer type* (Katzmann, 1976). It is a matter of controversy whether similar phenomenology alone is sufficient for grouping Alzheimer's disease and senile dementia of the Alzheimer type together. DSM-III-R bases the diagnosis of Alzheimer's disease and senile dementia of the Alzheimer type on specific criteria that render Alzheimer's disease/senile dementia of the Alzheimer type an exclusion diagnosis, only to be made if all other diseases that can cause mental impairment are absent. With the broadened definition now commonly used, age is the primary risk factor for Alzheimer's disease. The condition originally described as *presenile* dementia is thus a rare occurrence. Fewer than 5% of all Alzheimer patients belong to families where the early-onset disease seems to be inherited in an autosomal-dominant way (St. George-Hyslop et al., 1989). Among these families, a small subgroup has genetic mutations on chromosome 21 in the region that codes for the βA4-amyloid fragment of the amyloid precursor protein (APP), the protein containing the amyloid material of which the amyloid plaques are largely composed. Still, most of the families with familial Alzheimer's disease show no linkage to mutations in the APP gene. Further linkage evidence now implicates chromosome 19 (Roses et al., 1990) and chromosome 14 (Schellenberg et al., 1992). Degeneration of neurons and a neuropathological picture similar to Alzheimer's disease is seen in patients with trisomy 21 (Masters et al., 1985), supporting the importance of genetic factors. Nevertheless, most cases of Alzheimer's disease remain sporadic, and neither a known genetic cause nor a specific neuropathological marker exists.

NEUROPATHOLOGICAL CHARACTERISTICS OF ALZHEIMER'S DISEASE

Neurofibrillary Tangles

Neurofibrillary tangles (NFT) consist of ubiquitin-associated paired helical filaments of abnormally phosphorylated tau proteins (Grundke-Iqbal et al., 1986) reflecting the destruction of microtubules (Zhang et al., 1989) and neurofilaments (Matsuyama and Jarvik, 1989). These intraneuronal tangles appear to follow the formation of amyloid plaques and are likely to reflect neuronal damage, and eventually cell death (McKee et al., 1991). As reported by Alzheimer himself, the NFTs are not always present (Alzheimer, 1911). NFTs are regularly found in the pyramidal cells of the CA1 region of the hippocampus and in the superficial layers of the entorhinal cortex of nondemented elderly humans. In patients with fully developed Alzheimer's disease, the distribution of NFTs seems more widespread, including neurons of the layers III and V of the association cortex, the basal forebrain nuclei, and various brainstem nuclei. If extensive formation of NFTs has occurred, as in severe Alzheimer's disease, they can be found as extracellular residua. Since NFTs are present in various other diseases of the central nervous system, like progressive supranuclear palsy, Guam Parkinson dementia, and subacute sclerosing panencephalitis, they are not specific for Alzheimer's disease but rather the hallmark of neuronal cell death (for review see Mori and Ihara, 1991). Apart from NFTs, innumerable dystrophic neurites can be found in Alzheimer's disease tissue. They are often referred to as "curly fibers." Further exclusively neuronal hallmarks of nerve cell degeneration are the Hirano bodies and the granulovacuoles. Both structures are apparently remnants of condensed components of the destroyed neuronal cytoskeleton (Probst et al., 1991).

Senile Plaques

Senile plaques (Simchowicz, 1910) are spherical structures of 10 to 150 μm diameter. They lie in the extracellular space and are found in the brains of most elderly humans. The occurrence and density of senile plaques does not directly correlate with the degree of dementia (DeKosky and Scheff, 1990; Dickson et al., 1992). It seems that the clinically observed memory loss is correlated rather with the preferential localization of the neuropathological changes within the entorhinal cortex (Brodman area 28) of the ventrolateral temporal lobe and in areas of the hippocampal formation (Van Hoesen et al., 1991).

The main proteinaceous component of a senile plaque is the 4.2 kD βA4 amyloid protein that is part of a larger precursor, the amyloid precursor protein (Glenner and Wong, 1984; Masters et al., 1985; Kang et al., 1987; Müller-Hill and Beyreuther, 1989) (Figure 68-2). Apart from βA4, the senile plaques contain many proteins, like complement and the major histocompatibility complex (MHC) glycoproteins, that are otherwise characteristic for an inflammatory reaction. Senile plaques can be observed at various stages of their formation. Therefore, a number of morphological subtypes have been described.

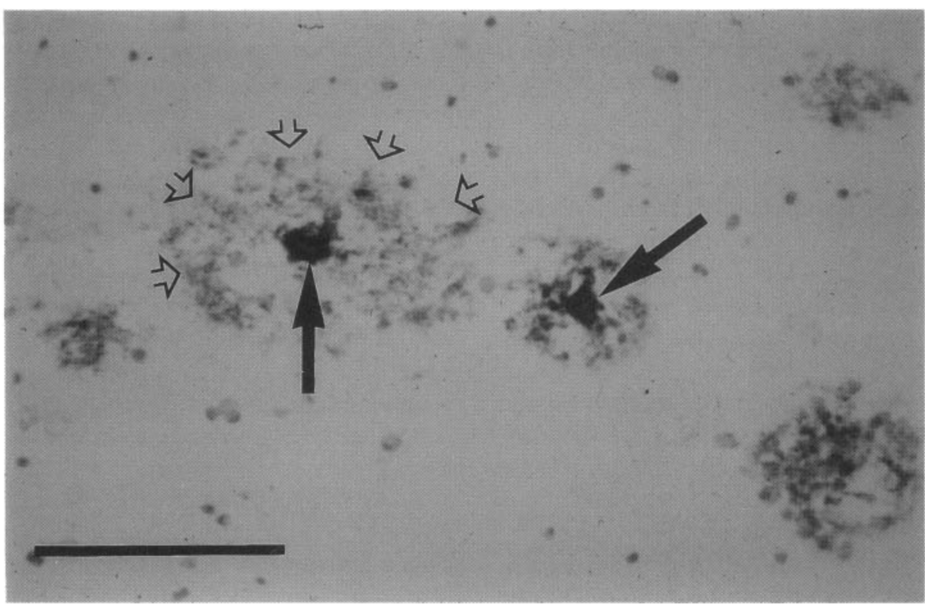


FIG. 68-2. Classical amyloid plaque. Staining with a monoclonal antibody against amyloid precursor protein (Weidemann et al., 1989) demonstrates that βA4 is the main proteinaceous component of the amyloid depositions in a classical amyloid plaque with its characteristic central core and the surrounding halo as described by A. Alzheimer. Scale bar = 150 μm.

According to Alzheimer's observation (Alzheimer, 1911), the formation of a plaque starts with a "Verdichtung des Grundgewebes" [condensation of tissue] leading to a "Hof ohne Kern" [halo of surrounding tissue without core]. Such a "Filzwerk" (meshwork]; (Braunmühl, 1957) constitutes a "Primitivplaque" [primitive plaque]; Braunmühl, 1957). This stage has also been designated "plaque A" (Probst et al., 1987) or "diffuse" (Yamaguchi et al., 1988) or "amorphous plaques" (Rozemuller et al., 1989). At this initial stage, no neuronal cell damage is observed, no amyloid or fibrillary structures are present, but the microglial cells as, "the early cellular response element of the brain" and "sensor to threat," already show the first signs of activation (McGeer et al., 1987; Probst et al., 1987; Streit et al., 1988; Gehrman et al., 1992; Gehrmann et al., 1993; Banati et al., 1993c). Upon careful examination, microglia are found to surround the pyramidal neurons that are about to die, a phenomenon Alzheimer and his pupils Simchowicz and Perusini referred to as building a microglial "Totenlade" [coffin] around the damaged neurons (Perusini, 1909; Simchowicz, 1910). With further disease progression, the next step is the formation of a primitive plaque (according to the definition of Wisnieski and Terry, 1973) in which the first fibrillary structures, composed of βA4 amyloid protein, appear. As an indicator of the severity of the pathological process, the activated microglial cells are now accompanied by reactive astrocytes with their characteristically enlarged cytoplasmic processes, originally described by Alzheimer as glial fibers. Very primitive plaques and diffuse plaques make up for the vast majority (>90%) of all plaques (Yamaguchi et al., 1988). Fewer than 10% have a dense amyloid core and are surrounded by a corona of swollen neurites which Alzheimer's described as "Höfe mit Kern" [halo with core]. They have now been designated "classical or typical plaques" (Wisniewski and Terry, 1973). A further very rarely seen variation consists entirely of compact amyloid (Alzheimer: "Kerne ohne Höfe," that is, core without halo) and is therefore called "compact plaque" (Wisniewski and Terry, 1973) (Figure 68-2).

MORPHOGENESIS OF SENILE PLAQUES AS AN EXAMPLE FOR NEUROGLIAL INTERACTION

From morphological observations, it has become clear that glial cells, namely, microglia and astrocytes, are actively involved in the formation of amyloid plaques. It is still an ongoing debate whether the origin of the amyloid is neuronal, vascular, i.e., from the bloodstream, or glial. The amyloid protein precursor (APP) can be produced by different cell types, including neurons, where it can be axonally transported (Shivers et al., 1988; Koo et al., 1990), astrocytes (Card et al., 1988; Neve et al., 1988; Banati et al., in press) and microglia (Wisniewski et al., 1989; Haass et al., 1991; LeBlanc et al., 1991; König

et al, 1992; Mönning et al., 1992). APP is now recognized to form a family of proteins. Its neuronal prototype, APP695, contains the structural domain of an integral transmembrane receptor (Kang et al., 1987). The different APP isoforms (Beyreuther and Masters, 1991; König et al., 1992) (Figure 68-3) are the result of differential splicing. The APP family falls into two categories: (1) the primarily neuronal isoforms (APP695) and (2) the primarily nonneuronal isoforms that contain a Kunitz protease inhibitor domain (Weidemann et al., 1989; Ohyagi et al., 1990).

Upon isolation of crude brain homogenate, the primarily neuronal APP695 is by far the most dominant of all APP isoforms in the central nervous system. In contrast to the relative abundance of the neuronal APP695, numerous investigations revealed that the APP isoforms increased in normal aging, Alzheimer's disease, and in experimental models, are mostly those containing the Kunitz protease inhibitor domain (Palmert et al., 1988; Tanzi et al., 1988; Siman et al., 1989; Tanaka et al., 1989; Golde et al., 1990, Johnson et al., 1990; Abe et al., 1991; König et al., 1991; Scott et al., 1991; Tanaka et al., 1992). This lends support to the hypothesis that either alternative splicing of APP in neurons is altered or that reactive glial cells contribute to the increased production of APP in aging and in the course of disease or experimental lesion. As a source for the amyloid deposited in close vicinity of blood vessels, specific cells of the mononuclear phagocyte system, the perivascular cells, seem to be a likely source (Graeber et al., 1989; Wegiel and Wisniewski, 1992) (Figure 68-6a). Oligodendrocytes, ependymal cells, mantle cells in spinal cord ganglia, and Schwann cells have also been reported to express APP (Karawabayashi et al., 1991).

As APP is principally a ubiquitous protein found in most cell types, the *sequence* in which different cells participate in different pathologies is of crucial importance in understanding the complex process of amyloid plaque formation. Based on the observation that microglia is usually the first cell to be activated in most pathologies of the central nervous system it

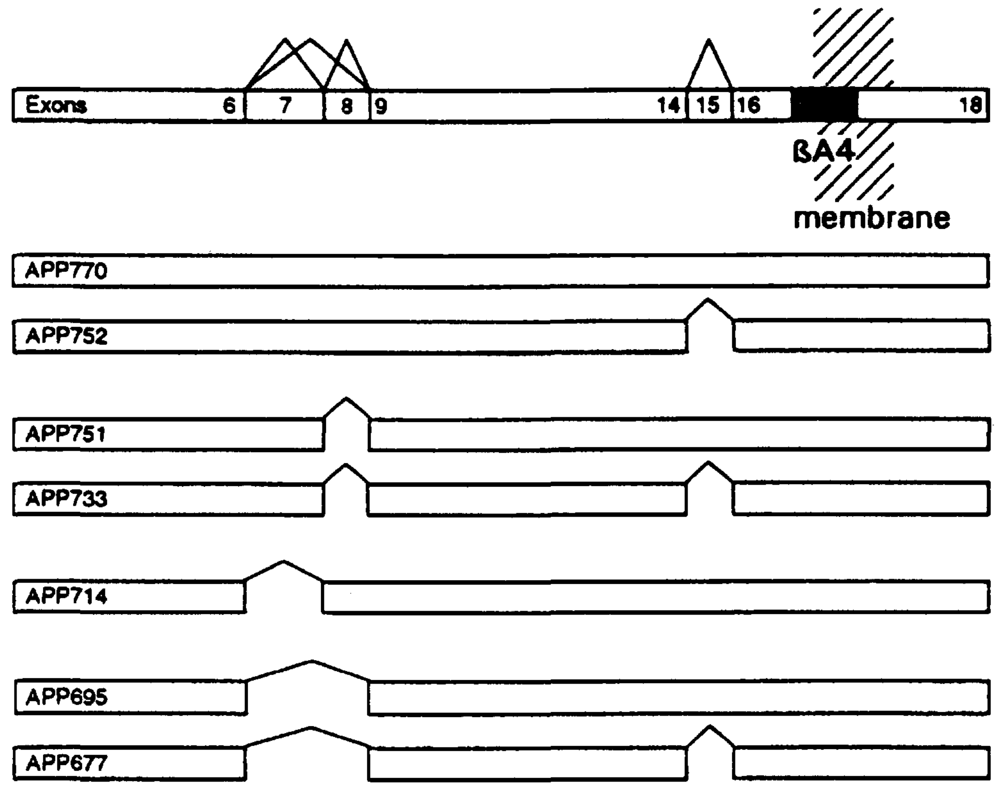


FIG. 68-3. Alternative splicing products of the *APP* gene. The amyloidogenic βA4 fragment lies near the C-terminal end of the amyloid precursor protein APP and is partly inserted into the cell membrane. The neuronal splicing forms lack the Kunitz protease inhibitor domain (i.e., Exon 7). In disease and experimental lesion the Kunitz protease inhibitor domain containing isoforms appear to be preferentially upregulated. (Exon 8 = OX 42).

has been suggested that the production of APP and subsequent amyloid deposition starts with an initial microglial response to neuronal dysfunction. Neuronal dysfunction might result either from external factors such as head trauma in the case of Morbus Alzheimer Pugilistica (Clinton et al., 1991), or intrinsic factors like increased neuronal vulnerability, possibly secondary to genetic factors. Once the production of APP is induced, its metabolic processing regularly leads to the release of βA4 (Haass et al., 1992), although it might represent only a minor portion among other APP cleavage products. Repeated and chronic induction, as might happen in the aging brain by a multitude of causes, could eventually lead to the accumulation of βA4 and by additional, yet to be clearly identified cofactors, to the formation of amyloid. Mutations in close proximity to the region encoding for βA4 in familial Alzheimer's disease have been taken as evidence that Alzheimer's disease is caused by an aberrant amyloidogenic processing of APP. Indeed, an increased production of βA4 has recently been reported for cell lines transfected with constructs bearing a mutation in the βA4 region (Citron et al., 1992; Cai et al., 1993). Whether the other known mutations in that region also cause an alteration of the metabolic pathway of APP remains to be shown. Cathepsin B is one of several enzymes that cleaves APP with high efficiency and is found in considerable amounts in activated microglia and within senile plaques (Cataldo and Nixon, 1990; Banati et al., 1993b). Free radicals are also regarded as important amyloidogenic cofactors promoting the aggregation of the βA4 amyloid (Dyrks et al., 1992). For proteolytic enzymes and free radicals activated microglia can be a potent source (Banati et al., 1991b, 1993b). Apart from proteolytic enzymes, microglia also contain protease inhibitors like cystatin C (Zucker-Franklin et al., 1987). A mutation of cystatin C is the cause of hereditary cerebral hemorrhage with amyloidosis (HCHWA) of Icelandic type (Ghiso et al., 1986). This example illustrates that glial cells are potentially important for the processing of APP. Amongst the proteins that have recently come into the focus of attention is apolipoprotein E (ApoE), certain isoforms of which appear to be associated with an increased risk for Alzheimer's disease. Interestingly, it is yet another protein that can be synthesised by cells of mononuclear-phagocyte lineage in response to nerve injury (Poirier, 1994). The early involvement of a cell type-like microglia that derives from mononuclear-phagocyte lineage (Banati et al., 1991a) would also explain the presence of the various inflammatory acute phase response proteins from the earliest stages of the disease (McGeer and Rogers, 1992; Strauss et al., 1992).

EXPERIMENTAL MODELS TO STUDY GLIAL AMYLOID PRECURSOR PROTEIN EXPRESSION

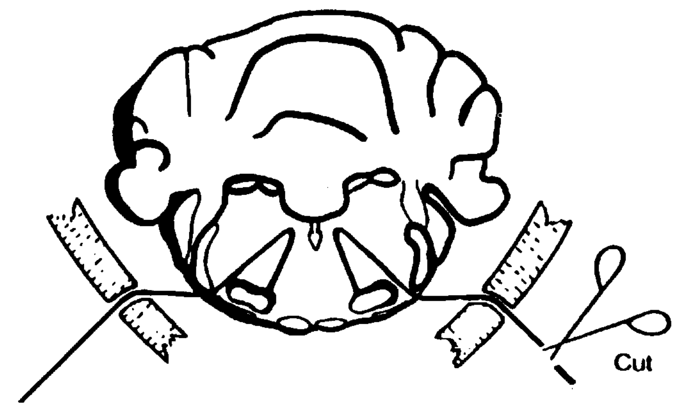
A number of models (for review see Beyreuther and Masters, 1991) have been established to study Alzheimer's disease under experimental conditions:

1. Aged nonhuman primates (Walker et al., 1990)
2. βA4 and APP mutant transgenics
3. Mouse ts16 transplant model (Richards et al., 1991)
4. PrP/scrapie Creutzfeld-Jakob disease, with plaques and perivascular amyloid (Prusiner et al., 1991)

The mechanism by which the amyloid precursor protein (APP) is converted to βA4, as well as its most relevant cellular source, is still mostly hypothetical. The following two subsections illustrate the regulation of APP in glial cells as one prerequisite for βA4 accumulation.

Peripheral Nerve Lesions

Acute activation of microglia can be stimulated in a graded fashion. One example is the transection of peripheral nerves such as the facial or sciatic nerve. Such nerve transections without damage to the blood-brain barrier cause a microglial reaction within hours in the nucleus of origin (Figure 68-4) or in projection areas in the central nervous system (Figure 68-7). Axotomy of the facial nerve prompts a rapid induction of APP immunoreactivity in microglial cells lying in close apposition to the injured neuron (Figures 68-4, 68-5, 68-6). Peripheral nerve lesions lead to a predominantly glial upregulation of APP mRNA and protein as early as 6 to 12 hours postlesion, not only at the site of affected neuronal cell bodies but also in corresponding projection areas (Banati et al., 1993a). The latter phenomenon can be observed after sciatic nerve transection when a glial APP induction can be seen in the nucleus gracilis, the projection area of the primary afferent fibers in the brainstem (Gehrmann et al., 1991) (Figure 68-7). Similarly, a lesion of the entorhinal cortex can induce glial APP-production in the dentate gyrus of the hippocampal formation (Banati et al., 1994a). The time course of APP-expression after sciatic nerve transection suggests a rapid transneuronal signaling to glial cells rather than axonal transport of APP to this area. In these experimental models light and electron microscopy demonstrates that microglia are a dominant source for nonneuronal APP expression.



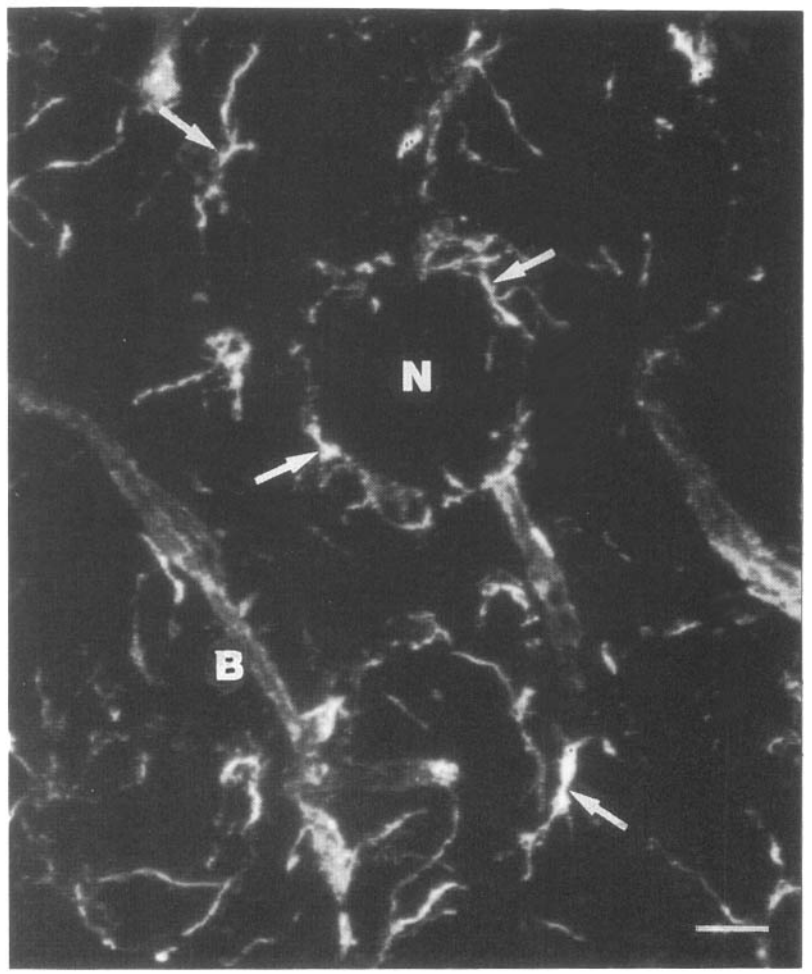


FIG. 68-4. APP immunoreactivity in microglia after facial nerve transection. Rat facial nerve axotomy is a lesion that leaves the blood-brain barrier intact and leads to a retrograde reaction in the motoneurons of the facial nucleus. Here, APP immunoreactivity in perineuronal microglia 4 days after lesion was detected with a monoclonal anti-APP antibody (Weidemann et al., 1989). The first APP immunoreactivity can be seen as early as 24 hours after lesion. APP is constitutively expressed on blood vessels. The picture was taken with a fluorescence confocal microscope (Leica; 40.0/1.3 oil, *xy*, 125 × 125 μm, extended focus). *N*, neuron; *B*, blood vessel. Scale bar = 25 μm.

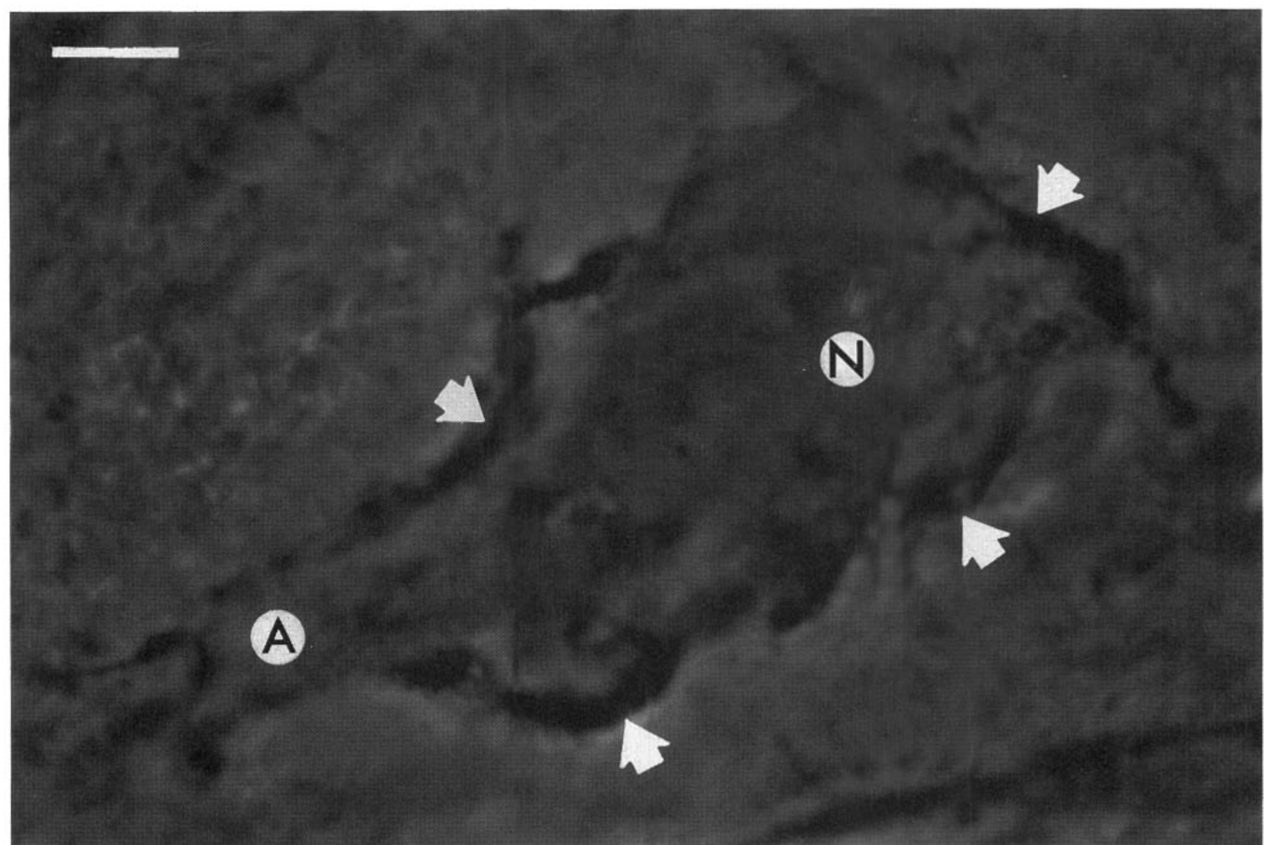


FIG. 68-5. APP immunoreactivity in perineuronal microglia. The axotomized motoneuron is closely ensheathed by strongly APP-immunoreactive microglia stained with a monoclonal anti-APP antibody (Weidemann et al., 1989). (Phase contrast picture taken 4 days after rat facial nerve axotomy.) *N*, neuron; *A*, axon. Scale bar = 10 μm.

Immune-Mediated Induction of Amyloid Precursor Protein

Microglia, which account for about 20% of all glial cells (Lawson et al., 1990), are capable of expressing marked levels of MHC class II. Upon activation, microglia rapidly upregulate the expression of MHC class II molecules (Gehrmann et al., 1992). Astrocytes can also express these immune molecules *in vitro* (Wekerle et al., 1986). Indicative of the involvement of immunocompetent cells in Alzheimer's disease is the presence of MHC class II molecules, which are regularly detected in amyloid plaques (Perlmutter et al., 1992).

A model that allows the study of glial APP synthesis in the context of a central nervous immune reaction is the experimental autoimmune encephalomyelitis (Banati et al., in press). Experimental autoimmune encephalomyelitis can be induced in naive syngeneic animals by the transfer of activated T cells, which recognize antigens of the central nervous system myelin sheath, such as myelin basic protein (Gehrmann et al., 1992). The disease is characterized at the histopathological level by the presence of inflammatory perivascular infiltrates, composed mainly of T lymphocytes, B lymphocytes, and macrophages. Glial cells, most conspicuously microglia, also respond to this inflammatory lesion by becoming activated. At an early preclinical stage (24 hours after T cell transfer) only very few glial cells newly express APP immunoreactivity. At the peak of clinical disease (6 days after T cell transfer), characteristic ramified cells are strongly positive for APP (Figure 68-8). Based on morphological criteria and double-labeling, the majority of these cells can be identified as microglia. Correlating with the course of clinical disease, the induction of APP in reactive microglia terminates in the postclinical stage, that is, 14 days after T cell transfer. These results indicate that APP can be induced in activated glial cells, particularly microglia, in immune-mediated disease.

CONCLUSIONS

The presence of many proteins related to the immune system and the dominant involvement of glial cells, particularly microglia, has led to various hy-

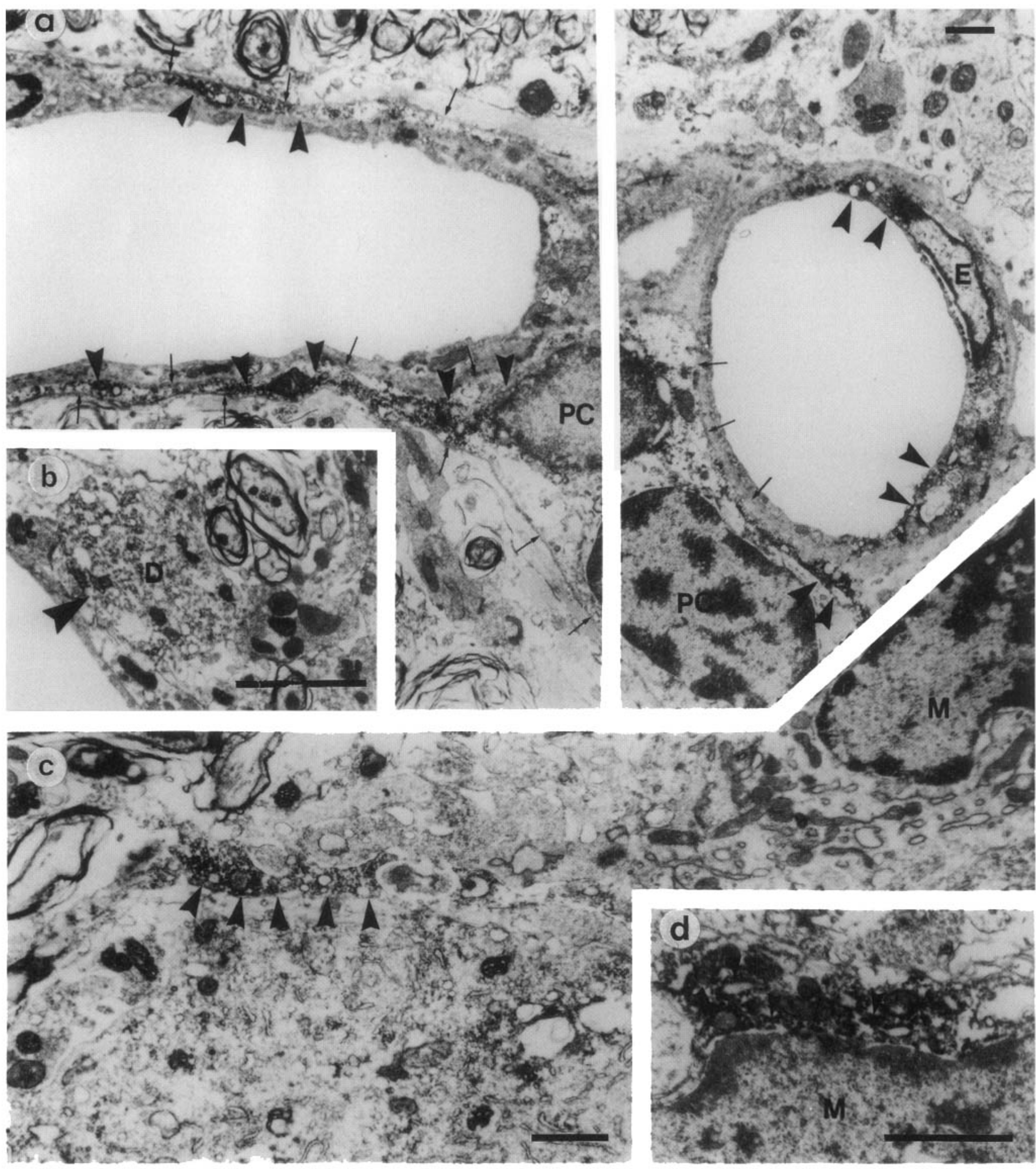
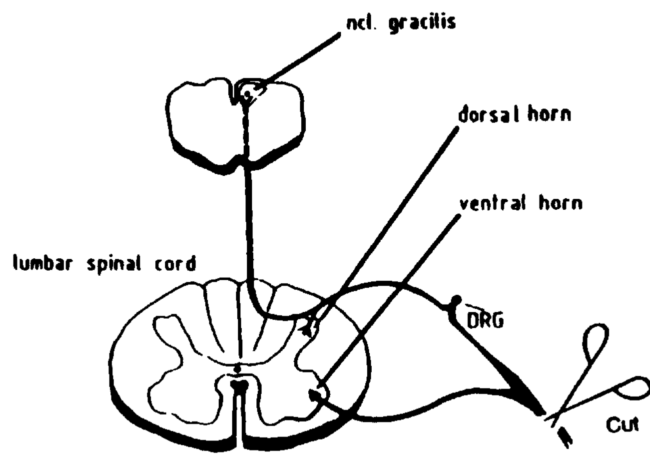
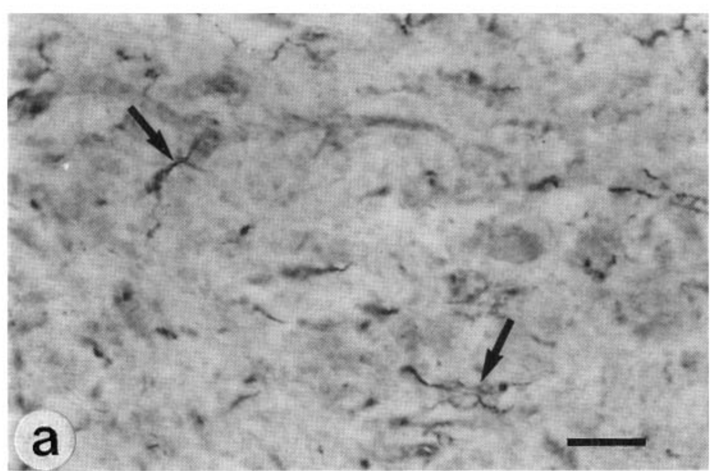


FIG. 68-6. Electron microscopic detection of APP. (A) In the control rat facial nucleus non-neuronal APP immunoreactivity (anti-APP antibody by Weidemann et al., 1989), as indicated by the dark DAB reaction product (*arrowheads*), is constitutively expressed in endothelial cells and in processes of perivascular cells, a cell type that lies within the basal lamina (*arrows*). *PC*, perivascular cell; *E*, endothelial cell. (B) The insert shows APP immunoreactivity in a dendrite (possibly bound to cytoskeletal proteins). *D*, dendrite. (C) 4 days after rat facial nerve axotomy-activated microglia show APP immunoreactivity in their cytoplasm (*c*) within the typically enlarged endoplasmatic reticulum (**d**). *M*, microglia. Scale bars = 1 μm.





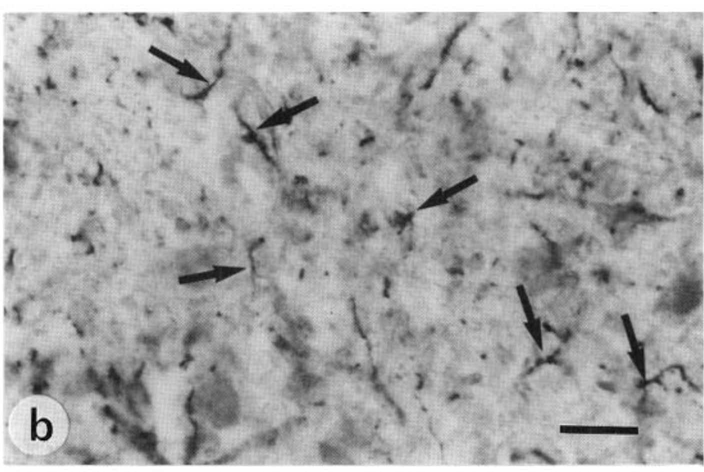


FIG. 68-7. Induction of APP immunoreactivity in microglia by anterograde neuronal reaction. The sciatic nerve sends primary afferent fibers into the nucleus gracilis. After induction by rat sciatic nerve transection APP immunoreactivity (anti-APP antibody by Weidemann et al., 1989) is light microscopically found in microglia as early as (A) 12 hours and more strongly after (B) 24 hours. Unlike after facial nerve axotomy, the microglial cells do not lie perineuronally, but are scattered in the sciatic projection area. The rapid appearance of microglial APP suggests that it has been *de novo*-synthesized in microglia rather than axonally transported and incorporated by phagocytosis. Scale bar = 25 μm.

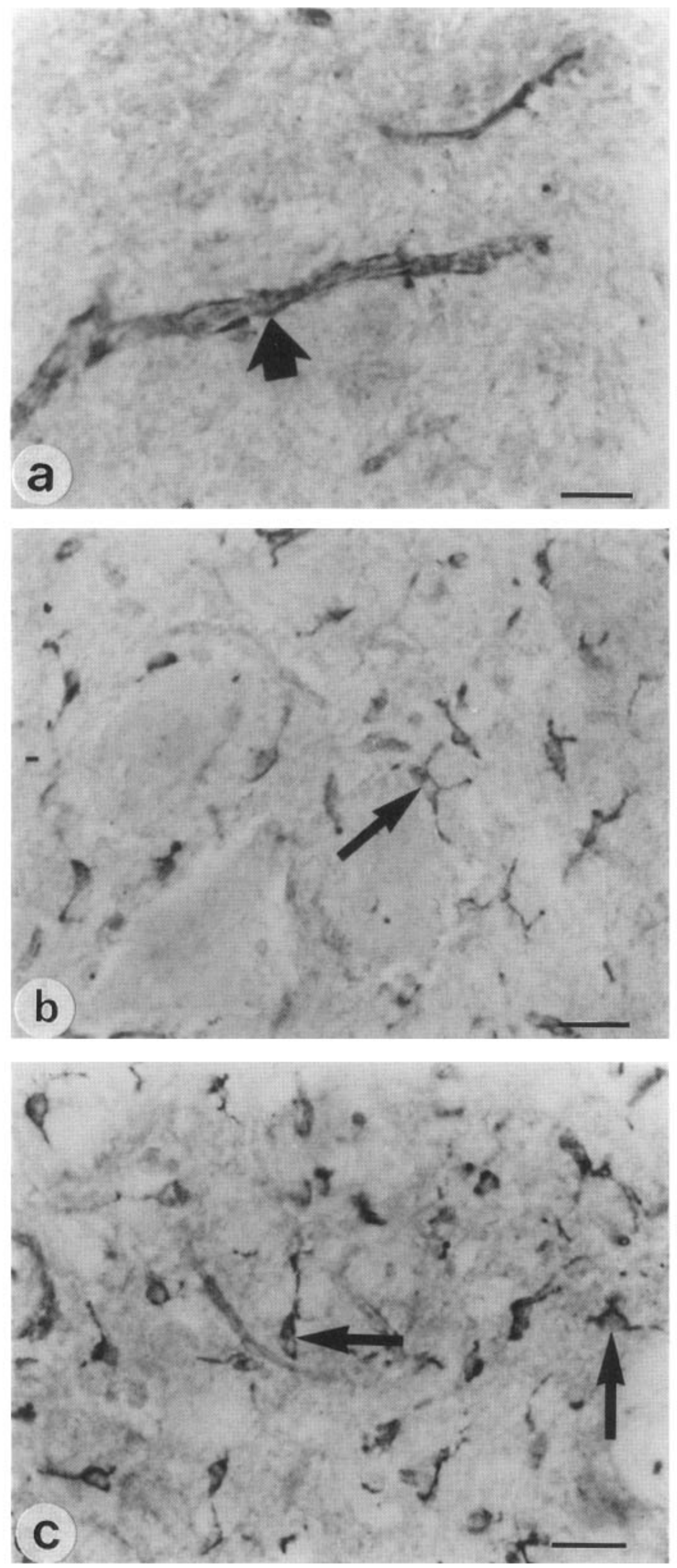
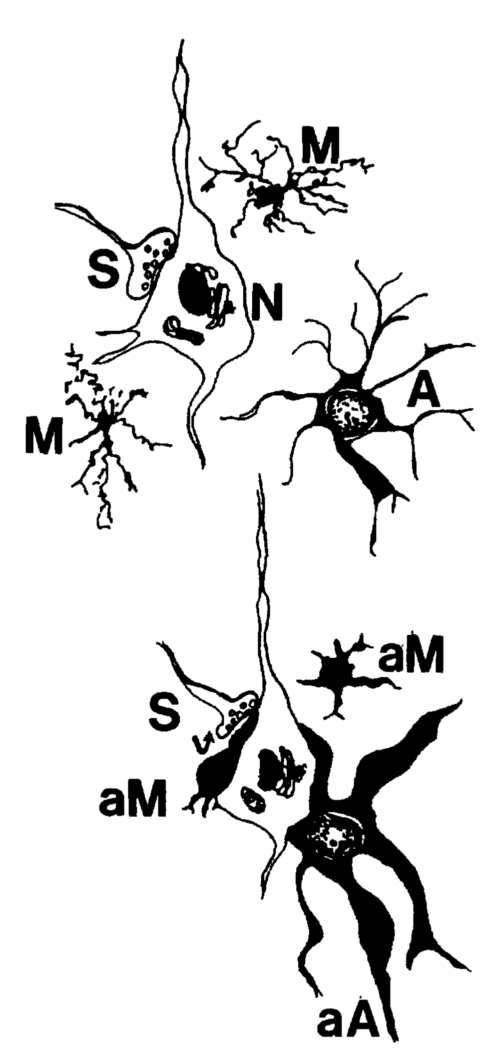


FIG. 68-8. Immune-mediated induction of APP. After transfer of activated T cells recognizing myelin basic protein into naive syngeneic rats, an experimental autoimmune encephalitis is induced. This causes an activation of microglia that goes along with a marked microglial expression of APP (as detected light microscopically by anti-APP antibody; Weidemann et al., 1989). The model indicates that also immune-mediated diseases can induce APP synthesis in glial cells. (**A**) Constitutive APP expression is seen in controls on blood vessels. The strongest APP immunoreactivity in microglia in the (**B**) anterior horn and the (**C**) dorsal horn of the spinal cord is seen between 6 and 7 days after T cell transfer, which correlates with the peak of clinical disease.



Gial reactions in response to neuronal lesion/dysfunction

A hypothetical model of amyloidogenesis

N	neuron	**S**	synapse
A	astrocyte	**aA**	activated astrocyte
fA	fibrous astrocyte	**rM**	resting microglia
aM	activated microglia	**pM**	phagocytic microglia
AP	amyloid plaque		

Early glial reactions

A variety of pathogenic stimuli can lead to neuronal reactions with subsequent glial activation. The cellular activation is accompanied by an up-regulation of APP expression in activated microglia and astrocytes. It is presently unclear in which cell type, neuron or glia, this stress-induced up-regulation of APP-synthesis mainly occurs. Activated microglia can perform ."synaptic stripping" leading to a de-afferentiation of the neuron (↯).

Chronic glial reactions

If the pathogenic stimulus persists microglia remain activated and the number of protoplasmatic astrocytes further increases. The dead neuron is eventually removed by brain macrophages/microglia. A continuously high synthesis of APP may overload the normal metabolic pathways and lead to the accumulation of ßA4-amyloid. In some cases of familiar Alzheimer's disease a mutation in the region coding for the ßA4 fraction might be instrumental in either increasing ßA4-amyloid production or altering its normal cleavage pathway.

Deposition of amyloid

With the cessation of the pathogenic stimulus microglia go back into their resting state and an astroglial scar remains. The amyloid plaque has formed during a chronic process that also led to the deposition of numerous other inflammatory proteins. It is an open question whether the diseased neuron itself could have produced the amyloid. In comparison, chronically activated glial cells do not only synthesise APP but also have a large repertoire of proteolytic enzymes and are capable of producing reactive oxygen intermediates, both pathophysiologically important amyloidogenic co-factors.

FIG. 68-9. Glial reactions in response to neuronal lesion or dysfunction.

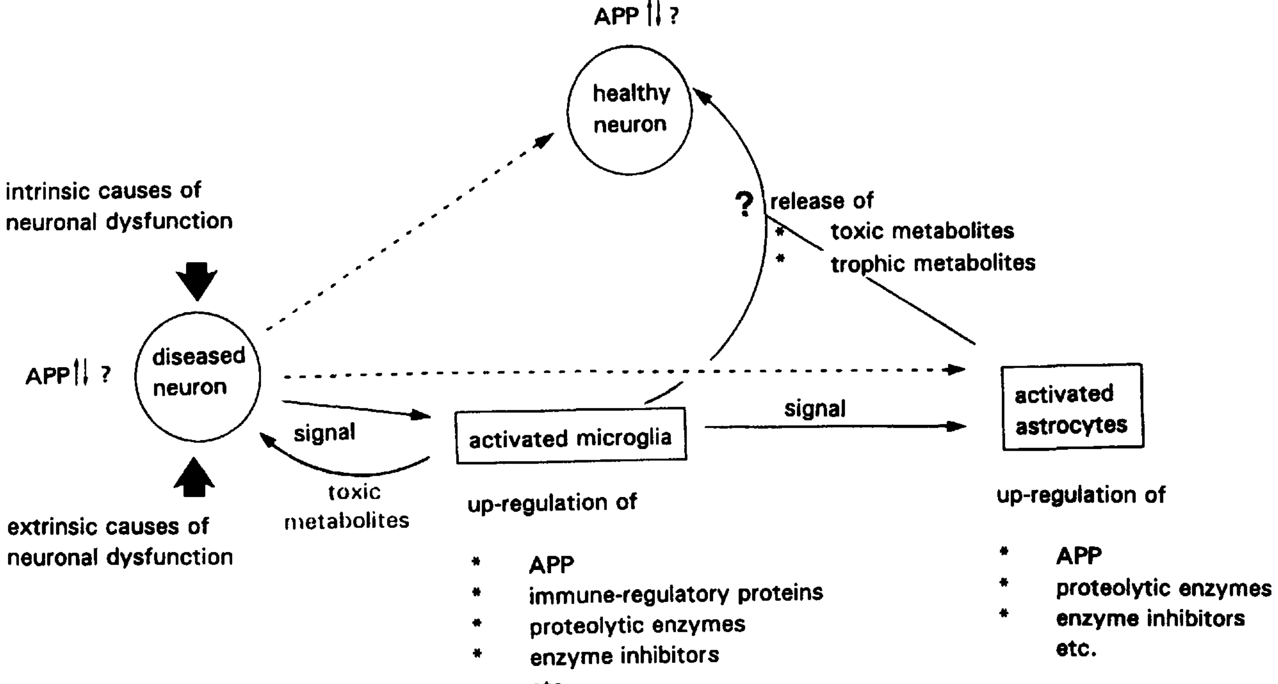


FIG. 68-10. Model of amyloidogenesis. The source of βA4 and how its precursor APP is regulated in neurons is still controversial. A number of studies, however, have shown that in Alzheimer's disease or in experimental lesions of the central nervous system the nonneuronal APP isoforms are increased. This implicates a dominant glial involvement in the formation of the amyloid plaque. Based on these observations, the model suggests that a dying or injured neuron acts on the local glia in its vicinity. The activation of glial cells goes along with the upregulation of APP. In the presence of other cofactors of amyloidogenesis, such as free radicals or APP proteolysis, βA4 might accumulate and eventually form the amyloid plaque. Such a glial activation might also have bystander effects on other neurons, for example, by the release of toxic (for review of cytotoxicity of microglia see Banati et al., 1993c) or trophic factors. The latter could explain the frequently seen sprouting of neurons into the plaque. The induction of APP in projection areas (as in the nucleus gracilis after sciatic nerve lesion, see Figure 68-7) might cause a cascade of similar events in areas of still healthy neurons. While the nature of the signal from neuron to microglia (and possibly also to astrocytes) is still open, the signal from microglia to astrocytes could be a member of the cytokine family (Giulian et al., 1988; Griffin et al., 1989).

potheses regarding the formation of senile plaques in Alzheimer's disease as part of an acute phase response (McGeer et al., 1987; Bauer et al., 1991; Nakajima et al., 1992). The function of APP in the immunopathology of Alzheimer's disease and related disorders is still largely unknown. Growth and differentiation factors like TGFβ can modulate the biosynthesis of APP in microglia depending on other factors like cell-cell or cell-extracellular matrix (Mönning et al., 1994). One hypothesis, therefore, is that APP could be important for the migratory behaviour of microglia, suggesting that the microglial APP synthesis is part of a physiological repair mechanism which under chronic stimulation might initiate the process of pre-clinical amyloid generation. As mentioned earlier, the number of senile plaques correlates imperfectly with the development of dementia. In contrast, decreased synaptic density gives a good measure for impaired cognitive functions (Masliah et al., 1989; DeKosky and Scheff, 1990). One characteristic function of activated microglia is neuronal deafferentiation by synaptic stripping (Blinzinger and Kreutzberg, 1968), a function they perform even after subtle neuronal injury. Chronic or repeated pathogenic stimuli acting upon the neurons, and the subsequent reactions of microglia and other glial cells, might thus eventually lead to a massive loss of synaptic connections (Figures 68-9 and 68-10). Such destruction/reconstruction of central nervous system tissue might involve toxic metabolites, like glutamate, nitric oxide or reactive oxygen intermediates, trophic factors, and tissue remodeling enzymes, that is, mechanisms that could be relevant for different therapeutic strategies (Abraham, 1989; Banati et al., 1993c).

Despite similar phenomenology of Alzheimer's disease and senile dementia of the Alzheimer type, it is still an open question whether these disorders belong to the same disease entity. Their etiology remains unknown. This chapter has therefore focused

on a description of the morphological changes seen in these conditions. The description of neuropathological phenomena evolving during the course of the human central nervous system disease or experimental models reveals that a variety of adverse noxious stimuli to the brain result in a common set of glial reactions. One of them is the rapid glial synthesis of APP, the protein that harbors the βA4, which itself is the main constituent of the senile plaque in Alzheimer's disease. Mutations of this protein in cases of familial Alzheimer's disease have underlined its importance. Therefore, these rare diseases now serve as models not only to study the pathophysiology of presenile dementia but also other forms of neuronal degeneration. The defining criteria of Alzheimer's disease, namely, clinical dementia and a certain neuropathology, have been questioned because of their limited specificity with respect to their cause. As happened with "schizophrenia" (Odegard, 1967; Frith, 1992), the perception and definition of "Alzheimer's disease" might therefore be subject to change.

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69 | Gliomas

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Brain tumors amount to less than 2% of all malignant neoplasms and thus constitute a small fraction of the overall human cancer burden. However, they exert a wide spectrum of adverse effects, and for most of them, the prognosis is still poor. Furthermore, a significant proportion of central nervous system neoplasms affect children, in whom tumors of the nervous system (including retinoblastomas and peripheral neuroblastomas) rank second in incidence after leukemias. Brain tumor biology has for a long time been a *terra incognita*, but in recent years considerable progress has been made, particularly in the immunology and molecular genetics of gliomas. In addition, angiogenesis in human brain tumors and the biological basis of the migratory potential of neoplastic glial cells have become a focus of attention.

EPIDEMIOLOGY AND ETIOLOGY OF GLIOMAS

Analytical epidemiological studies show considerable geographical variation in the incidence of brain tumors, which generally tends to be higher in developed, industrial countries (Parkin et al., 1992). In Western Europe and North America, there are about 5 to 9 new cases of primary central nervous system tumors per 100,000 inhabitants each year. Approximately half of these are of glial origin. In multiracial countries, whites are more frequently affected than persons of African or Asian descent. In our study of 1330 patients (Figure 69-1), the ratio of male to female patients was 1.4:1, in accordance with other reports. This contrasts with the more benign meningiomas, which occur predominantly in women (Schiffer, 1993). There has been some controversy about a possible increase in the incidence of brain tumors over the past two decades, particularly in the elderly (Boyle et al., 1990; Greig et al., 1990; Davis et al., 1991). Most authors agree that, at least in part, this apparent increase is due to the introduction of high-resolution neuroimaging (computed tomography, magnetic resonance imaging), which has greatly improved the clinical diagnosis of neurological diseases (Desmeules et al., 1992; Modan et al., 1992).

With the exception of inherited neoplastic syndromes (e.g., neurofibromatosis, von Hippel-Lindau disease, tuberous sclerosis, germline mutations of the *p53* tumor suppressor gene), the etiology of human brain tumors is largely unknown (Schoenberg, 1982). Analytical epidemiological studies have revealed an increased risk of brain tumor development in association with certain occupations (T. Thomas and Waxweiler, 1986), for example, farmers, dentists, fire fighters, metal workers and rubber industry workers, but attempts to identify a specific exposure or causative environmental agent have been unsuccessful. The somewhat increased incidence of central nervous system neoplasms in anatomists (Stroup et al., 1986), pathologists (Harrington and Oakes, 1984) and embalmers (Walrath and Fraumeni, 1984) points to a possible role of formaldehyde, but it has emerged that in an industrial setting exposure to this weak carcinogen is not associated with an increased risk (Stroup et al., 1986). Similarly, multicenter cohort studies have not substantiated the hypothesis that occupational exposure to vinyl chloride carries an enhanced risk of brain tumor development (Simonato et al., 1991). This is also true for electromagnetic fields and environmental or medical (Schlehofer et al., 1992) radiation exposure. However, therapeutic irradiation of the brain, particularly in children, may lead to the development of malignant gliomas or primitive neuroectodermal tumors after an interval of usually 6 to 9 years (Brüstle et al., 1992b). A causative link between glioma development and previous neurological diseases (head trauma, epilepsy) has been suspected but remains unproven. This is also true for parental exposures to potential carcinogens and previous medical histories of various types during pregnancy (Schiffer, 1993).

PATHOLOGY AND CLINICAL ASPECTS

As in other tissues, the classification of tumors of the central nervous system is based on histogenetic principles, that is, the medical terminology relates to the

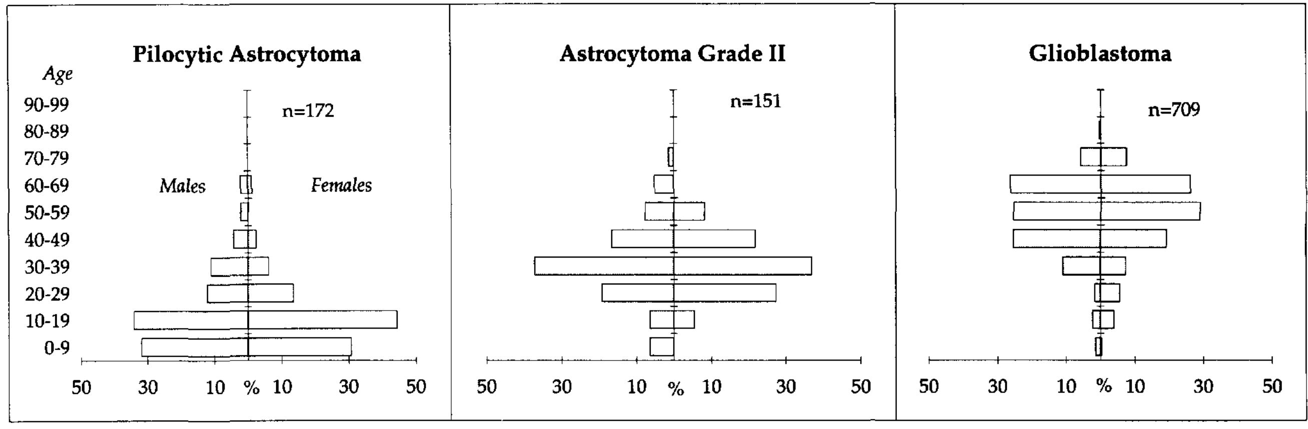


FIG. 69-1. Age and sex distribution of patients with gliomas (Institute of Neuropathology, University of Zürich, 1975–1993). Note that the age peak of malignant gliomas occurs much later than that of their benign counterparts.

cell of origin. Although gliomas are assumed to be derived from only three cell types, that is, differentiated astrocytes, oligodendrocytes and ependymal cells, the respective neoplasms display a large spectrum of different histopathological features, which in turn are associated with marked differences in incidence, location within the central nervous system, preferential age of affected patients and clinical course and outcome. It is now generally accepted that this variation in the phenotype and biological behavior of gliomas largely reflects the type of transformation-associated genes operative in neoplastic development.

Neurological symptoms resulting from glioma development depend primarily on the site of the tumor. However, the length of the patient's history and the chances for a long, remittance-free survival are more closely associated with the neoplasm's intrinsic biology, in particular, its proliferation potential, its inherent tendency for progression to anaplasia and its capacity to rapidly invade the surrounding brain tissue. For practical purposes, these characteristics are usually condensed into a grading scheme (Table 69-1). While the histogenesis of a neoplasm can often be derived from its morphological features and immunoreactivity profile, the biological correlates, in particular the clinical course and outcome, can only be estimated by comparisons with clinical data from patients with similar lesions. Therefore, in the grading of gliomas anaplasia is used as an operational term summarizing histopathological features typically associated with malignant biological behavior and may include nuclear atypia, increased mitotic activity, atypical mitoses, cellular pleomorphism, vascular proliferation, and necrosis. The significance of some of these features varies considerably in different tumor types. For example, nuclear atypia and extensive vascular profileration indicate anaplasia and a poor clinical prognosis in diffuse astrocytomas of the cerebral hemispheres but have no such implications in a pilocytic astrocytoma.

Astrocytic Tumors

Astrocytomas are tumors composed predominantly of neoplastic astrocytes. If not stated otherwise, this term applies to diffusely infiltrating neoplasms, which, according to their biological behavior, are subdivided into low-grade astrocytoma (WHO Grade II), anaplastic astrocytoma (WHO Grade III), and glioblastoma multiforme (WHO Grade IV) (Kleihues et al., 1993a,b).

Low-Grade Astrocytoma. Typically, low-grade astrocytomas occur in young adults, with a peak incidence in the fourth and fifth decades. They may be located in any region of the central nervous system, including the spinal cord but their preferential location is in the cerebral hemispheres, where they rep-

TABLE 69-1. *Grading of Astrocytic Tumors*

WHO definition	Grade	Features
Pilocytic astrocytoma	I	Rosenthal fibers, piloid cells
Low-grade astrocytoma	II	Nuclear atypia
Anaplastic astrocytoma	III	Mitotic activity
Glioblastoma multiforme	IV	Vascular proliferation/ necrosis

resent approximately 25% of all gliomas (Schiffer, 1993). Clinical symptoms may vary from several months to years according to location. Postoperative survival is typically from 3 to 5 years and depends largely on whether or not the tumor progresses to anaplasia and more rapid growth. Due to the infiltrative nature of these neoplasms, permanent cure through complete surgical resection is extremely rare. Macroscopically, low-grade astrocytomas are ill-defined, yellow-white, and homogeneous (Figure 69-2A). Single or multiple cysts containing a clear fluid may be seen. Diffuse infiltration often leads to enlargement and distortion, but not destruction, of the invaded anatomical structures, for example, cortex and compact myelinated pathways. Mitotic activity is absent. Microscopically, astrocytomas show a consistent tendency to diffusely infiltrate into the surrounding brain. Preexisting cell types, for example, neurons, are often entrapped. Phenotypically, neoplastic astrocytes may vary considerably in size, prominence, and disposition of cell processes, and abundance of cytoplasmic glial filaments. According to the prevailing cell type, the following variants can be distinguished.

Fibrillary Astrocytoma. By far the most frequently occurring astrocytoma is the fibrillary variant. Cell density is low to moderate. Nuclear atypia may be prominent. The cytoplasm is often scant and barely discernible. In more cellular lesions, numerous cell processes form a loose fibrillary matrix, often with microcyst formation (Figure 69-2B). Glial fibrillary acidic protein (GFAP) is consistently expressed in such areas.

Protoplasmic Astrocytoma. The protoplasmic astrocytoma is a rare variant characterized by neoplastic astrocytes with a small cell body and few flaccid processes with a low content of glial filaments. Accordingly, GFAP immunostaining is scant. Microcyst formation is common.

Gemistocytic Astrocytoma. Another variant of astrocytoma is predominantly composed of gemistocytic astrocytes. Tumor cells show large eosinophilic cell bodies, an angular shape, and stout, randomly oriented, GFAP-positive processes forming a coarse fibrillary network (Figure 69-2C). Nuclei are usually eccentric. Perivascular lymphocyte cuffing is frequent.

Anaplastic (Malignant) Astrocytoma. Focal or diffuse anaplasia, for example, increased cellularity, pleomorphism, nuclear atypia, and mitotic activity, characterizes the anaplastic or malignant astrocytoma. GFAP immunostaining may vary. Anaplastic astrocytomas show an inherent and often rapid tendency to progress to glioblastoma.

Glioblastoma. Glioblastoma is the most frequent and malignant brain tumor, and usually affects adults, with a peak incidence between 45 and 60 years. It is typically located in the cerebral hemispheres, most frequently involving the frontal and temporal lobe. The clinical history is usually short (less than 3 months) unless the neoplasm has developed from low-grade or anaplastic astrocytoma (secondary glioblastoma). Patients typically present with unspecific neurological symptoms, headache, and personality changes, but most threatening is the rapid development of raised intracranial pressure. After incomplete surgical resection and radiotherapy, the mean survival time is in the range of 6 to 9 months.

Macroscopically, glioblastomas are poorly defined, with varied coloration due to degeneration, necrosis, and occasional hemorrhage (Figure 69-3A). Histologically, they are composed of poorly differentiated, fusiform, round, or pleomorphic cells and occasional multinucleated giant cells. The presence of prominent vascular proliferation and/or necrosis is essential for the histological diagnosis (Figure 69-3B). The latter may comprise more than 80% of the total tumor mass. Mitotic activity and GFAP expression vary considerably. Many glioblastomas contain areas with differentiated neoplastic astrocytes, indicating their astrocytic nature and frequent evolution from low-grade or anaplastic astrocytoma (Burger and Kleihues, 1989). Two variants of glioblastoma are recognized. The giant cell glioblastoma is characterized by a predominance of bizarre, multinucleated giant cells and, on occasion, an abundant stromal reticulin network (Figure 69-3C). The gliosarcoma contains a neoplastic sarcomatous component, which is presumed to originate from a malignant transformation of the hyperplastic vascular elements typically observed in glioblastomas (see below).

Pilocytic Astrocytoma. After the embryonic central nervous system neoplasms, the pilocytic astrocytoma is the most frequent brain tumor in childhood, with a peak incidence around the age of 10 years. In contrast to the diffuse astrocytic tumors, the pilocytic astrocytoma is a more circumscribed, slowly expanding lesion, which very rarely shows a tendency to progress to anaplasia and therefore corresponds to WHO grade I (Kleihues et al., 1993a). Pilocytic astrocytomas are typically located in midline structures, that is, the optic nerve (optic glioma), the third ventricle, the thalamus, the median temporal lobe,

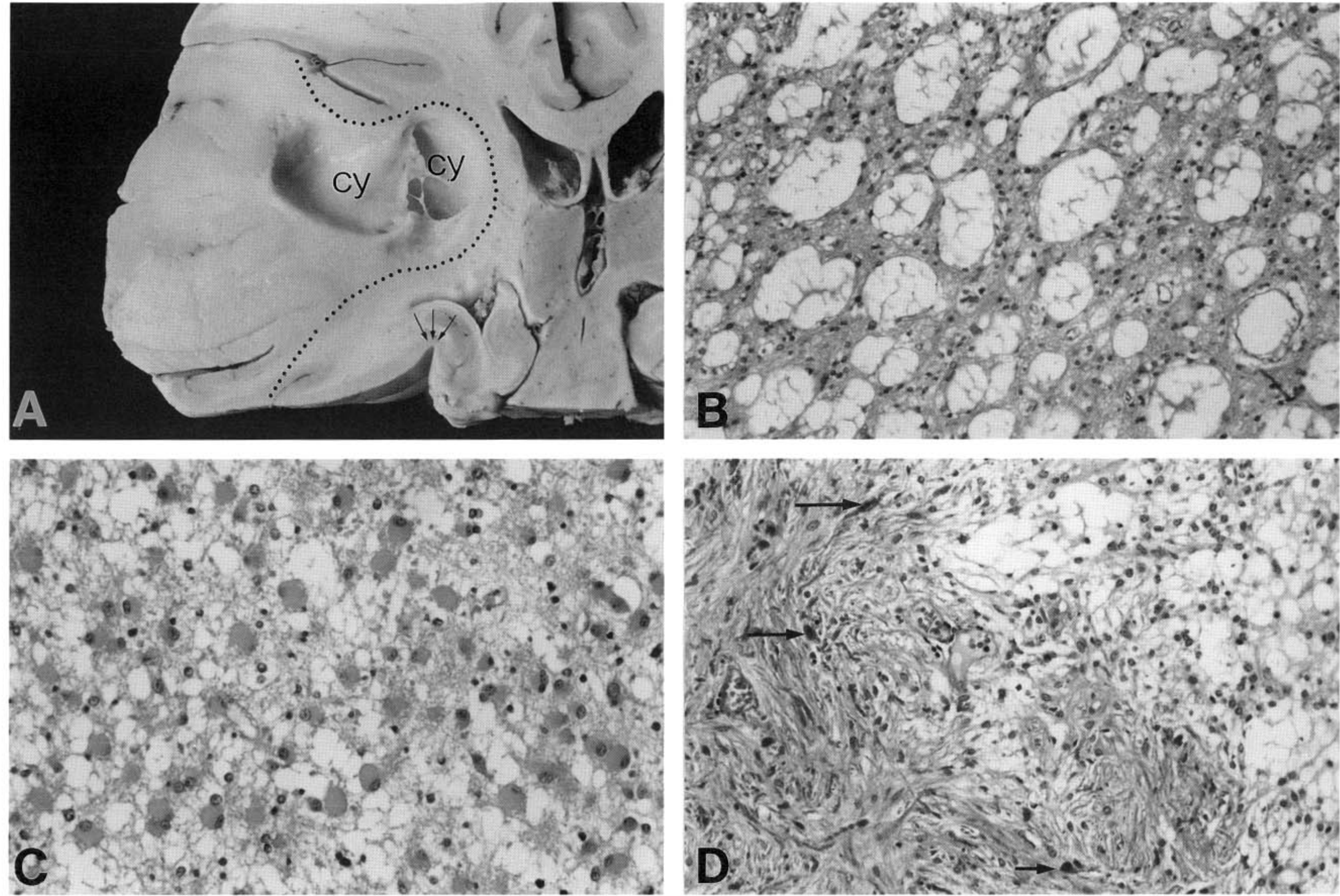
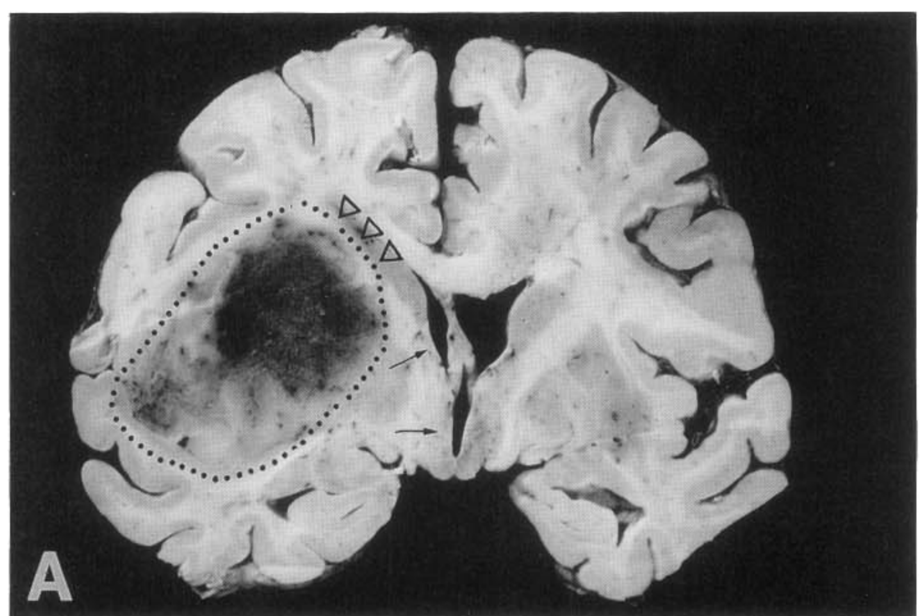
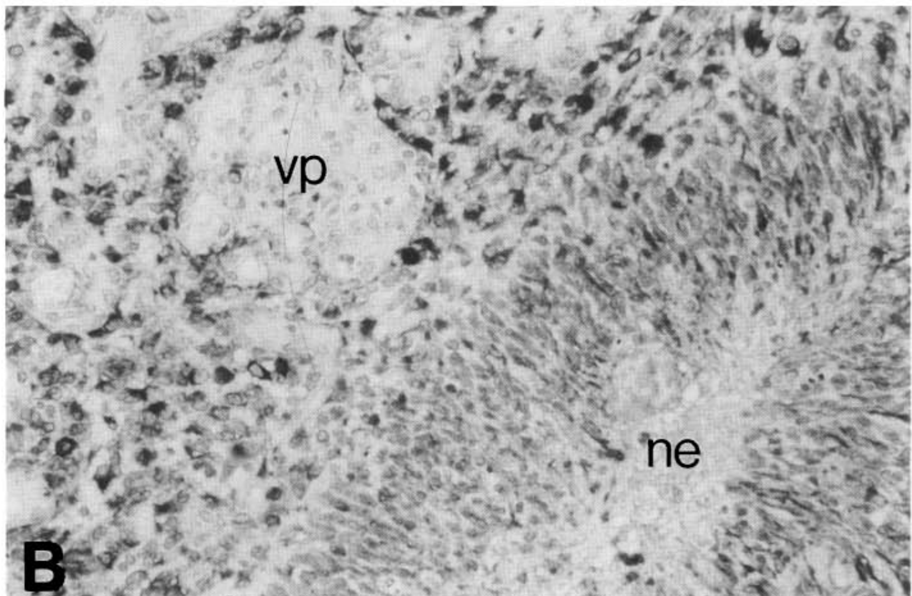


FIG. 69-2. Gross pathological and histological appearance of low grade astrocytomas. (**A**) Coronal section through the brain of a patient who died from astrocytoma of the left temporal lobe. The whitish tumor with large cysts [CY and ill-defined borders (*dotted line*)] diffusely infiltrates temporal gyri and the white matter. Although this neoplasm did not show histological signs of malignancy, it caused transtentorial herniation (*arrow*) and compression of the brainstem. (**B**) Typical histological appearance of a fibrillary astrocytoma with microcystic degeneration. (**C**) Gemistocytic astrocytoma with large cells showing a swollen cytoplasm, short thick processes, and eccentric nuclei. (**D**) Pilocytic astrocytoma featuring the typical biphasic pattern of fibrillary areas containing Rosenthal fibers (*arrows*) and loosely textured areas with microcystic degeneration.





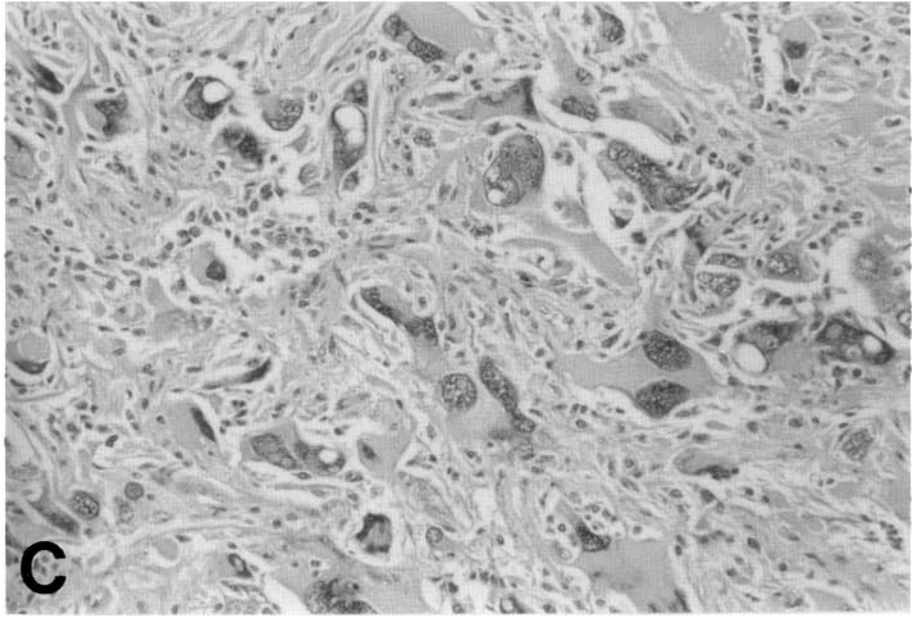


FIG. 69-3. Glioblastoma multiforme. (**A**) Coronal section showing a glioblastoma of the left cerebral hemisphere with hemorrhagic and necrotic components. The borders with the adjacent brain are ill defined (*dotted line*). Invasion of the capsula interna (*CI*) had caused hemiplegia. Note the compression of the ipsilateral ventricle (*arrow*). (**B**) Typical histological appearance of the glioblastoma with extensive vascular proliferations (*VP*) and necrosis (*NE*), surrounded by "pseudopalisading" tumor cells (GFAP immunostain). (**C**) Clusters of bizarre shaped, often multinucleated tumor cells in a giant cell glioblastoma.

the brainstem and the cerebellum. Total surgical resection is difficult, with the exception of cerebellar lesions. The clinical course often extends over many years, but eventually most tumors recur.

Macroscopically, the pilocytic astrocytoma is homogeneous, often cystic, and is well circumscribed. It is histologically characterized by bipolar, fusiform or "piloid" cells with dense fibrillation. It is particularly common to see a biphasic pattern in which pilocytic areas are intimately associated with a loosely structured microcystic component. Hyperchromatic bizarre nuclei, endothelial proliferation and invasion of the meninges are common but do not signify malignancy. Elongated eosinophilic, club-shaped structures (Rosenthal fibers) and eosinophilic intracytoplasmic protein droplets ("granular bodies") are histopathological hallmarks of this neoplasm (Figure 2D). GFAP expression is always demonstrable, although to a varying degree.

Pleomorphic Xanthoastrocytoma. The variant of astrocytoma known as pleomorphic xanthoastrocytoma occurs characteristically in children or young adults and affects the cerebral hemispheres, particularly the temporal lobe. It is typically superficial in location, shows an intimate relation to the meninges and may be associated with an underlying cyst. Histological hallmarks are pleomorphic tumor cells, which range from ordinary fibrillary astrocytes to giant, multinucleated forms. The latter typically contain lipid vacuoles; thus they are xanthomatous, but express GFAP. Pleomorphic xanthoastrocytomas are usually well demarcated and can often have a favorable prognosis (Kepes, 1993).

Subependymal Giant Cell Astrocytoma. The circumscribed, benign tumors known as subependymal giant cell astrocytomas often overlie the head of the caudate nucleus and typically occur in young patients as part of the tuberous sclerosis complex. They arise from the walls of the lateral ventricles, and are typically calcified. They are composed of large, plump cells that resemble astrocytes. GFAP expression is highly variable. These lesions grow very slowly and can be resected surgically when secondary complications, such as occlusion of the foramen of Monro, occur.

Oligodendroglial Tumors

Oligodendrogliomas usually affect adults and develop slowly over a period of years, often with seizures as the only clinical manifestation (WHO Grade II). They are preferentially located in the cerebral white matter and basal ganglia, are macroscopically pink and, in contrast to astrocytomas, tend to invade the leptomeninges. Tumor cells typically exhibit spherical, often hyperchromatic nuclei surrounded by artifactually swollen, clear cytoplasm. A well-defined plasma membrane lends them a so-called fried egg appearance. The stroma consists mainly of delicate, capillary-sized blood vessels. Focal calcifications in the peripheral zone of infiltration are a histopathological hallmark of oligodendrogliomas. No specific immunocytochemical marker is available for these tumors. A fraction of otherwise typical neoplastic oligodendrocytes expresses GFAP. Progression to anaplastic oligodendroglioma may occur, but is less frequent than in diffusely infiltrating astrocytomas.

Ependymal Tumors

Ependymomas develop from the ependymal or subependymal cells surrounding the ventricles and the central canal of the spinal cord, as well as from ependymal clusters in the filum terminale. Intracranial ependymomas occur predominantly in children and tend to fill the ventricular lumen. Clinical symptoms are usually due to blockage of cerebrospinal fluid flow, which may lead to a sudden increase in intracranial pressure. Slowly growing and usually well-delineated from adjacent brain structures, ependymomas correspond histologically to grade II. Spinal ependymomas are usually diagnosed in adults and are preferentially located in the lumbosacral region.

Histological hallmarks include perivascular pseudorosettes and ependymal rosettes. GFAP expression varies considerably and, if present, is usually restricted to the radiating cell processes of perivascular pseudorosettes. The more aggressively growing anaplastic ependymoma (WHO Grade III) is mainly characterized by brisk mitotic activity.

Among various histological variants only the *myxopapillary ependymoma* is of interest, as it occurs almost exclusively in the region of the cauda equina and originates from the filum terminale. Tumor cells may also surround pools of mucin, but their glial nature can usually be confirmed by immunoreactivity for GFAP. These neoplasms grow very slowly and correspond to WHO Grade I.

Subependymoma. The slowly growing benign tumors (WHO Grade I) called subependymomas appear as well-delineated, occasionally multiple firm nodules attached to the wall of the fourth or the lateral ventricles. They rarely obstruct cerebrospinal fluid pathways but are otherwise asymptomatic and typically detected at autopsy. Microscopically, sub-

ependymomas consist of clusters of ependymal cells with round nuclei, surrounded by broad swirls of glial processes. Despite their usually slow growth, a fraction of subependymomas causes symptoms by obstructing the cerebral aquaeduct.

PROLIFERATION AND GROWTH FRACTION

Growth and extension of a neoplasm depend on a variety of factors, the most important ones being their proliferation potential, the extent of cell loss and the ability of tumor cells to invade adjacent tissues and to metastasize. Gliomas have a considerable capacity to diffusely infiltrate the brain but penetration through the arachnoidal space is less common and metastasis to distant tissues is a very rare event. Most studies on the rate of proliferation of human gliomas have been carried out with BrdU immunostaining (Gratzner, 1982) or with the monoclonal antibody Ki-67 (Gerdes et al., 1984; Gerdes et al., 1992). The former method determines the fraction of tumor cells in the S-phase of the cell cycle (analogous to thymidine) but requires an infusion of BrdU in patients prior to or during surgical intervention (Hoshino et al., 1986). The monoclonal antibody Ki-67 can only be used on frozen sections while the more recently developed antibody MIB-1 can be applied to archival, formalin fixed, paraffin-embedded tissues (Cattoretti et al., 1992; Gerdes et al., 1992). These antibodies recognize a nuclear antigen expressed in all continuously cycling cells, that is, during G_1, S, G_2, and M phases of the cells cycle but not in resting cells (G_0 phase). Thus, the fraction of labeled cells directly reflects the size of the growth fraction. Accordingly, the BrdU labeling index (LI) is usually somewhat lower: LI BrdU = 0.34 × LI Ki-67 + 0.99 (Morimura et al., 1989). In gliomas, the labeling index generally agrees with histological grade and clinical behavior of the different tumors. In the benign, juvenile pilocytic astrocytoma, the LI is usually lower than 1%. For the diffuse infiltrating low-grade and anaplastic gliomas, the mean Ki-67 LI is typically 1 to 2% and 3 to 5%, respectively (Burger et al., 1986; Shibata et al., 1988; Zuber et al., 1988; Raghavan et al., 1990). Data for the glioblastoma vary considerably and can be higher than 30%. Since these neoplasms often show extensive necrosis, the proliferative fraction alone is not a valid criterion for tumor growth. The labeling index does not therefore correlate with individual patient survival in most studies (Bookwalter et al., 1986). More recent investigations seem to indicate that astrocytomas with a high Ki-67 LI, elevated *p53* protein and EGF receptor amplification are associated with malignant progression and poor prognosis (Jaros et al., 1992; Torp et al., 1992).

MIGRATION AND INVASION OF GLIOMA CELLS

It has long been realized that glioma cells migrate far beyond the apparent tumor border (Scherer, 1940a, 1940b). This tendency can be observed in low-grade as well as in high-grade astrocytomas, but is particularly noticeable in glioblastoma multiforme. Diffuse infiltration of the adjacent brain structures is the main reason that surgical resection is often incomplete, and even hemispherectomy may not prevent glioma recurrence (Burger et al., 1991). Anatomical structures vary in their susceptibility to glioma invasion. The glioblastoma extends very rapidly along compact myelin pathways, for example, corpus callosum, anterior commissure, fornix, optic radiation. Once tumor cells gain access to any of these structures, they rapidly migrate into the contralateral hemisphere. For this reason, glioblastomas frequently appear as multifocal tumors (Burger et al., 1988a). Rapid spread through the corpus callosum often leads to the macroscopic appearance of a lesion that unfolds symmetrically from the corpus callosum into the two hemispheres ("butterfly glioblastoma").

The migratory capacity of glioma cells can be reproduced in the rat brain, using either established rat glioma cells lines (Bernstein et al., 1990) or xenografted human glioma cells (Bernstein et al., 1989a, 1989b). As in the human brain, the corpus callosum has been identified as a major migration route. Further, grafted human astrocytes were found on the glia limitans at the outer surface of the brain, in the Virchow-Robin spaces and in the subependymal regions of the ventricles (Bernstein et al., 1989a). Another model system extensively investigated is the cocultivation of glioma spheroids and reaggregated fetal brain cells (Lund et al., 1990b). Of the various factors which are potentially involved in glioma invasion, extracellular matrix proteins have received particular attention. There is emerging evidence that integrin, fibronectin, and laminin modulate tumor spread (Letourneau et al., 1988; Bjerkvig et al., 1989). A comparative immunohistochemical study on human brain tumors indicates that the expression of tenascin, but not fibronectin, correlates with angiogenesis and the degree of invasion and malignancy (Higuchi et al., 1993). The question as to whether human glioma cells produce and secrete tenascin or whether they induce its synthesis by mesenchymal cells, is still unresolved. Of the cell adhesion molecules, neural cell adhesion molecule

(NCAM) expression was investigated in two rat glioma lines which differ greatly in their metastatic ability. It was found that failure of BT4Cn cells to synthesize NCAM is associated with an increased metastatic potential (Andersson et al., 1991).

Several proteinases have been implicated in the migration of transformed glial cells within the central nervous system, including collagenase type IV (Taylor et al., 1991; Vaithilingam et al., 1991; Reith and Rucklidge, 1992), endopeptidase (Monod et al., 1989) and progelatinase (Miazaki et al., 1992). As in other tissues, metalloproteinases are prime suspects among enzymes facilitating tumor invasion. Several of these have been partially identified (Paganetti et al., 1988; Apodaca et al., 1990; Lund et al., 1991). A motility factor produced by malignant glioma cells has been postulated and partially characterized by Ohnishi et al. (1990). Finally, it should be noted that growth factors typically expressed in malignant gliomas (e.g., epidermal growth factor) may also contribute to the spread of tumor cells within the central nervous system (Lund et al., 1990a, 1992; Engebraaten et al., 1993).

ANGIOGENESIS IN GLIAL TUMORS

A large body of anatomical observations and of experimental evidence points to the critical importance of an efficient blood supply for the growth of malignant tumors (Folkman, 1990; Weidner et al., 1991; Folkman, 1992). This fact is particularly obvious in the case of tumors belonging to the astrocytic lineage. Low-grade astrocytomas, which are associated with a protracted and relatively benign clinical course, invariably show vascular densities similar to that of normal cerebral tissue (Feigin et al., 1958). In contrast, disordered vascular hyperproliferation is one of the most prominent hallmarks of malignant astrocytic tumors, that is, anaplastic astrocytoma and glioblastoma multiforme (Kleihues et al., 1993). It has been shown that the presence of vascular proliferation correlates positively with an unfavorable clinical course (Daumas et al., 1988). Pathological angiogenesis in high-grade astrocytomas characteristically affects the endothelial compartment of the microvascular tree and leads to formation of kinky corkscrewlike capillaries with grotesquely thickened walls (Figure 69-3C).

The extent to which vascular proliferations colonize malignant astrocytomas is rarely seen in other human neoplasms and has prompted speculation that glioblastomas might produce angiogenic factors. Indeed, basic and acidic fibroblast growth factors (bFGF and aFGF) have been identified in glioblastomas (Zagzag et al., 1990; Stefanik et al., 1991). The demonstration within pathological vascular proliferations of the production of platelet-derived growth factor by glioma cells and of its cognate receptors by endothelial cells provided an elegant framework for the concept of an autocrine angiogenetic loop in gliomas (Heldin et al., 1988; Heldin and Westermark, 1991; Plate et al., 1992a,b).

Recently, an additional angiogenetic system has been discovered in gliomas: the vascular endothelial growth factor (VEGF) and its cognate receptors, the *flk*-1 and *flt* tyrosine kinases. VEGF is a heparin-binding growth factor with target-cell specificity restricted to vascular endothelial cells (Leung et al., 1989; Ferrara et al., 1991). What sets VEGF apart from related growth factors, such as basic and acidic FGF, is its exquisite specificity for vascular endothelia. The group of Werner Risau has demonstrated strong transcription of VEGF by glioblastoma cells (Plate et al., 1992). Most intriguingly, VEGF mRNA as detected by *in situ* hybridization was specifically expressed by "pseudopalisading" glioma cells, that is, those cells located in the immediate neighborhood of necrotic areas.

In an elegant, synchronous study, Shweiki et al. (1992) reported similar results and asked the additional question whether the dramatic upregulation of VEGF transcription in pseudopalisading cells might be the immediate consequence of hypoxia. Exposure to hypoxia of cultured astrocytoma cells resulted in strong induction of the VEGF message, and surprisingly it was found that this induction, far from being restricted to glioma cells, was detectable in cultured cells as diverse as skeletal muscle myoblasts and in fibroblastic cell lines. The physiological importance of the angiogenic stimulus elicited by VEGF is underlined by the recent finding that inhibition of VEGF production *in vivo* effectively reduces the tumorigenic potential of injected transformed cells (Kim et al., 1993).

In an effort to identify receptor tyrosine kinases potentially involved in angiogenetic signal transduction, Millauer et al. (1993) identified various candidate molecules using degenerated polymerase chain reaction cloning primers from early postimplantation embryos and cultured endothelial cells. This interesting cloning strategy resulted in the realization that a tyrosine receptor kinase which had been independently identified shortly before (Matthews et al., 1991) and had been termed *flk*-1 (fetal liver kinase), is strongly and widely expressed in the endothelial lining of budding capillaries during embryogenesis. *Flk*-1 binds to VEGF with high affinity and can therefore be regarded as a true receptor for this growth factor. The interaction between VEGF

and *flk*-1 is likely to be important for the vascular hyperplasia seen in gliomas. An additional receptor for VEGF is *flt*, a member of the platelet-derived growth factor receptor tyrosine kinase family.

One still poorly understood aspect is the histogenesis of secondary neoplastic transformation of the mesenchymal component occasionally seen in glioblastoma (so-called gliosarcoma). It has been proposed that the malignant mesenchymal component in gliosarcoma is derived from transformation of endothelial or pericytic cells, although derivation from fibrohistiocytic elements has also been discussed (Grant et al., 1989). In view of the extraordinarily concentrated efforts currently invested in the clarification of angiogenetic mechanisms of glial tumors, it seems likely that we will soon reach conclusive answers to these questions.

MOLECULAR GENETICS

It is now generically accepted that cancer cells evolve through a multistep process of malignant transformation, which genotypically is characterized by the sequential acquisition of genetic alterations. The latter includes the activation of protooncogenes and the inactivation of tumor suppressor genes. Several of these steps have recently been identified in human brain tumors.

p53 Tumor Suppressor Gene

The *p53* tumor suppressor gene is located on the short arm of chromosome 17 and encodes a nuclear phosphoprotein involved in the regulation of cell proliferation (Boyd, 1990). The wild-type *p53* gene acts as a tumor suppressor gene, whereas some *p53* mutations occurring within highly conserved regions not only cause loss of tumor suppressor function but may convert the *p53* protein into a dominant oncogene (Eiyahu, 1989; Finlay, 1989). Various human tumors show a loss either of both alleles of the *p53* gene, the loss of one *p53* allele with an associated point mutation, insertion, or deletion of the remaining allele, or an inactivation of the *p53* gene in one allel but a normal (wild-type) sequence in the other. The rapidly accumulating data on *p53* genetic alterations indicate that it may constitute the gene most frequently involved in human oncogenesis (Hollstein, 1991; Levine, 1991). The function of the *p53* gene product appears to lie in the stabilization of the genome after damage by carcinogens and irradiation. These environmental DNA damaging agents cause a nuclear accumulation of *p53*, which leads to cell cycle arrest or apoptosis (Lane, 1992). In contrast, interference with the *p53* pathway leads to continuous cell division in the presence of promutagenic DNA lesions and, thus, stable somatic mutations. Inactivation of the *p53* gene may occur by mutation or complex formation with viral or cellular oncoproteins (Levine, 1991).

***p53* Mutations in Human Gliomas.** The occurrence of *p53* mutations is largely restricted to diffusely infiltrating astrocytomas and to glioblastoma multiforme (Ohgaki et al., 1993). In low-grade astrocytomas (WHO Grade II) *p53* mutations, with or without loss of heterozygosity on chromosome 17p, are the only detectable genetic alteration and occur at an overall incidence of 24% (Fults et al., 1992; von Deimling et al., 1992; Ohgaki et al., 1993). Anaplastic astrocytomas contain p53 mutations at an incidence of 32% (Fults et al., 1992; von Deimling et al., 1992a). In three independent studies, 24 *p53* mutations were identified in a total of 72 glioblastomas, that is, in 33% of cases (Frankel et al., 1992; Fults et al., 1992; Louis et al., 1993). The observation that *p53* mutations are present at a similar incidence in low-grade astrocytoma, anaplastic astrocytoma, and glioblastoma multiforme may indicate that this gene is involved during a rather early stage of neoplastic transformation. One study suggests that the progression to glioblastoma is characterized by a clonal expansion of cells carrying a *p53* mutation, presumably due to selective growth advantage (Sidranski et al., 1992). This view is supported by a report by Hayashi et al. (1991) who found *p53* mutations only in progressive recurrent gliomas. However, both studies are based on relatively few cases and require confirmation in a larger series of tumors. *p53* Mutations in astrocytomas are often associated with loss of heterozygosity of chromosome 17p (Frankel et al., 1992; von Deimling et al., 1992).

p53 mutations in astrocytic human brain tumors are predominantly located at cytosine-guanine repeats (CpG sites) (55%), as in a variety of other human neoplasms (Levine, 1991), including colon carcinoma, sarcomas, lymphomas/leukemias, bladder carcinoma, and small-cell lung carcinoma. The *p53* mutations were located in highly conserved regions with slight clusters at codons 175 (7.5%), 248 (17.5%), and 273 (7.5%) (Ohgaki et al., 1993). These three codons have also been reported to be hot spots for mutations in colon cancer (Levine, 1991). Among the types of *p53* mutations in these astrocytic brain tumors, G:C → A:T transition mutations prevail (56%), followed by frameshift mutations (18%). G → A transitions are also the most frequent type of *p53* mu-

tation in small cell lung cancer, colon cancer, and lymphoid malignancies (Hollstein, 1991).

In some gliomas and extraneural tumors, the wild-type p53 protein accumulates in gliomas, probably through increased half-life rather than overexpression (Finlay et al., 1988). The mechanisms underlying this phenomenon are still poorly understood. Brain tumors occur in approximately 12% of patients affected by the Li-Fraumeni syndrome (Malkin et al., 1990; Srivastava et al., 1990). This inherited neoplastic disease is often, though not invariably, caused by *p53* germline mutations. Other neoplasms associated with this syndrome are soft tissue and bone sarcomas, breast cancer, leukemia, and adrenocortical carcinomas.

In contrast to diffusely infiltrating astrocytomas, the juvenile pilocytic astrocytoma does not contain mutations in the highly conserved regions of the *p53* gene, suggesting a different genetic basis for this benign childhood neoplasm (Ohgaki et al., 1993). This is also largely true for nonastrocytic gliomas, that is, oligodendrogliomas and ependymomas (Ohgaki et al., 1991).

p53 Inactivation by Complex Formation with Oncoproteins. The *p53* protein can be inactivated by complex formation with oncoproteins of DNA viruses, including the large T antigen of the SV40 and polyoma viruses, E1A of adenovirus 2 and 5, and E7 of human papilloma virus 16. In addition, inactivation may occur through binding to the murine double minute 2 gene (*mdm2*). The human homologue of this gene is frequently amplified in childhood sarcomas and a similar mechanism appears to be operative in less than 10% of human anaplastic gliomas and glioblastomas (Reifenberger et al., 1993). In these cases, *p53* mutations are typically absent.

Growth Factors and Growth Factor Receptors

Several growth factors have been implicated in the evolution of human gliomas, including platelet-derived growth factor and its receptor (Eva et al., 1982), tumor necrosis factor β (de Martin et al., 1987; Constam et al., 1992) and insulinlike growth factor (IGF-1) (Sandberg et al., 1993). Most prominent is the involvement of the epidermal growth factor receptor (EGFR), which occurs in up to 50% of glioblastomas but are very rare in low-grade astrocytomas (WHO Grade II), and in anaplastic astrocytomas. Almost two-thirds of the glioblastomas with amplified EGFR genes also show coamplification of rearranged EFGR genes and concomitant expression of mRNA species (Libermann et al., 1985; Bigner et al., 1988; Bigner and Vogelstein, 1990; Ekstrand et al., 1991). Expression of EGFR with an isolated extracellular truncation including the ligand binding domain can induce transformation *in vitro* (Haley et al., 1989).

Chromosomal Loss in the Pathogenesis of Gliomas

In isolated cases of low-grade and anaplastic astrocytomas, loss of heterozygosity on chromosomes 9p, 13, and 22 have been reported. In addition, more than 40% of anaplastic astrocytomas show loss of heterozygosity on chromosome 19q (von Deimling et al., 1992). Furthermore, the involvement of a second yet unidentified tumor suppressor gene on chromosome 17p distinct from the *p53* gene has been suggested for malignant astrocytomas (Frankel et al., 1992; Saxena et al., 1992). Loss of chromosome 10 was found in one-fifth of anaplastic astrocytomas and more than 70% of glioblastoma multiforme (Fults et al., 1992; von Deimling et al., 1992b). All glioblastomas with EGFR amplification also show loss of chromosome 10 (von Deimling et al., 1992b).

Genetic Models of Glioma Progression

The sequence of gene alterations acquired during glioma development is shown in Figure 69-4. However, this has to be considered provisional since only

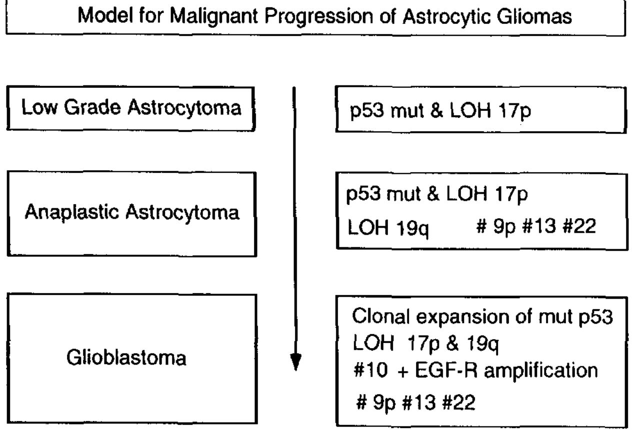


FIG. 69-4. Synopsis of genetic alterations associated with the evolution and progression of human gliomas. Mutations of the *p53* tumor suppressor gene, often in conjunction with loss of heterozygosity on chromosome 17, constitute the earliest detectable genetic change. Loss of chromosome 10 and amplification of the epidermal growth factor receptor (*EGF-R*) are largely restricted to the glioblastoma. [Modified from Ohgaki et al. (1993), with permission.]

a few transformation-associated genes have been identified so far. Nevertheless, there seems to be an emerging sequence of molecular events, with *p53* mutations being the first detectable alteration. The glioblastoma multiforme may develop *de novo* from differentiated astrocytes, usually with a short clinical history (primary glioblastoma). However, this lesion may also develop through progression from low grade and anaplastic astrocytoma (secondary glioblastoma). Based on molecular genetic analyses, von Deimling et al. (1993) have proposed two types of glioblastoma, one being characterized by loss of heterozygosity of chromosomes 17p and 10, the other by loss of heterozygosity of chromosome 10 and amplification of the EGFR gene. It remains to be shown whether this distinction correlates with differences in tumor phenotype and clinical behavior.

IMMUNOLOGY OF GLIOMAS

A large body of evidence indicates the existence of a complex network of interactions between neoplasms and the immune system of their hosts. In particular, the question has been pursued as to whether and to what extent tumors can trigger a cytotoxic immune reaction that may impair, with varying efficiency, their growth. It has also emerged that certain tumors may develop strategies aimed at paralyzing the immune system of the host, thereby optimizing their chances of expansion. Intriguingly, in glial tumors a good case can be made for both phenomena (Fontana et al., 1992). Several reports have pointed out that glioblastoma patients tend to suffer from a state of partial immune suppression. Downregulation of cutaneous immune reactivity, diminished numbers of T cells and marked depression of lymphocyte responses to lectins and antigens have been described and all point to impaired T cell-mediated immunity (Roszman et al., 1991). It remains to be ascertained whether this apparent downregulation of certain functions of the immune system modifies the biological behavior of the tumors in any clinically significant way.

Several exogenous factors and side effects of anticancer therapy might contribute to the decreased immunoreactivity. Treatment with antiepileptic drugs, especially hydantoins used to control tumor-related seizures, has been found to interfere with T cell immunity *in vivo* and *in vitro*. Corticosteroids used to reduce the glioma-associated brain edema are a well-established cause of decreased cellular immunity through the inhibition of cytokine secretion, of chemotaxis, and of expression of adhesion molecules involved in immune interactions. Furthermore, psychological stress, malnutrition, and, last but not least, specific tumor treatment with cytostatic agents and irradiation may contribute to impaired immunity in glioblastoma. As be discussed below, glioblastoma cells may even secrete immunosuppressive molecules that can hinder immune reactivity at the site of tumor growth.

Adriano Fontana and coworkers (1984) have shown that glioblastoma cells do not secrete a thymocyte-stimulatory factor, but rather inhibitory molecules blocking cytotoxic responses in primary mixed lymphocyte reactions. This factor, which has been termed glioblastoma-derived T cell suppressor factor (GTSF), was biochemically purified and shown to be a member of the family of transforming growth factor (TGF). In more recent studies, the same authors have characterized the elements controlling transcription of this factor in glioblastoma cells (Wrann et al., 1987; Schreiber et al., 1990). Anti-TGF-β2 antibodies neutralize the immunosuppressive activity detected in the supernatant of glioblastoma cells, indicating that at least *in vitro*, TGF-β2 is the only immunosuppressive factor that is released. TGF-β2 suppresses the secretion of TNF in mixed lymphocyte cultures, the production of H_2O_2 by macrophages stimulated with phorbol esters, the generation of lymphokine-activated killer cells, and the activation of natural killer cells. Furthermore, TGF-β2 influences the growth, differentiation, and immunoglobulin production of B lymphocytes (Wahl et al., 1989), although the molecular mechanisms leading to these effects are still poorly understood.

Although gemistocytic astrocytomas and glioblastomas are typically infiltrated to a varying extent by inflammatory cells, the efficiency of the immune response against gliomas might be compromised by the active inhibition of local immune responsiveness due to tumor-derived suppressor factors. Indeed, tumor-infiltrating T cells harvested from glioblastoma seem to be functionally arrested in a frozen state, and their expansion *in vitro* is almost impossible (Miescher et al., 1988). It has recently been suggested that escape from this type of negative control may be achieved by the loss of TGF-β binding sites on the surface of lymphocytes (Siepl et al., 1991).

One additional factor that may be rate-limiting in determining the biological effects of the above-mentioned system is the conversion of latent (L-) TGF-β to its active form. This process seems to be mediated by various cellular proteases and endoglycosidases (Huber et al., 1992). Treatment of glioblastoma cells with protease inhibitors may partially abolish the TGF-β-dependent inhibition of T cell growth (Huber et al., 1991) by interfering with the production of biologically active TGF-β. On the

other hand, a number of *in vitro* and *in vivo* findings point to the distinct possibility that glioma cells may have the potential to be a preferential target of immune surveillance.

Amid these contrasting viewpoints it is intriguing to note that neoplastic glial cells do share features typical of professional antigen presenting cells, such as expression of class I and II histocompatibility antigens (Carrel et al., 1982). Expression of histocompatibility antigens and of intercellular adhesion molecule (ICAM-1) is enhanced by interferon-γ, and such enhancement of expression seems to have functional importance as interferon-γ-treated glioma cells are efficient stimulators in the mixed lymphocyte reaction (Takiguchi et al., 1985). In addition, cultured glioma cells have been shown to express a wide variety of different cytokines, such as interleukin-1 and interleukin-6 (Takiguchi, et al., 1985; van Meir et al., 1990), granulocyte/macrophage colony stimulating factor (GM-CSF) and (G-CSF) (Tweardy et al., 1990; Frei et al., 1992). The significance of the expression of these growth factors and cytokines by the tumors *in situ*, however, has not yet been established with any certainty. Finally, glioblastoma cells may express immunologically important adhesion molecules such as ICAM-1, a cell adhesion molecule important for interaction with the leukocyte function associated antigen-1 (LFA-1, ICAM-1) (Kuppner et al., 1990).

The discovery of these peculiar immunological properties of neoplastic glial cells has triggered a wealth of studies aimed at their clinical exploitation. For example, Bigner and colleagues (1988) have characterized a monoclonal antibody termed Mel-14, which reacts with a chondroitin sulfate proteoglycan antigen in gliomas and other neoplasms. Intravenous administration of ^{131}I-labeled antibody significantly improved the survival of athymic mice transplanted intracerebrally with human glioma xenografts, thus suggesting the feasibility of radioimmunotherapy trials (Colapinto et al., 1990). In addition, the same group has shown that this antibody might be useful as a radioimaging reagent (Garg et al., 1992).

Generally, most attempts at using immunity to combat glial tumors had disappointing results (Apuzzo and Mitchell, 1981; Mahaley et al., 1983). A randomized clinical trial has shown no benefit from immunotherapy (Bloom et al., 1973). On the other hand, another clinical trial has suggested that survival can be increased if levamisole (an inhibitor of alkaline phosphatase) and BCG are added to irradiated homologous glial cells (Mahaley et al., 1983).

Recently, a very promising method has been developed, which exploits the combined antigenic properties and the requirements for autocrine growth factors for tumoral vaccination against gliomas. Initially, Trojan and colleagues (1992) reported that rat glioblastoma cells lose their tumorigenicity if production of insulinlike growth factor I (IGF-I) is inhibited by introducing antisense expression vectors. These results suggest that IGF-I is an important factor codetermining the malignant behavior of glioma cells *in vivo*. Based on the observation that histological sections revealed only a few glioma cells infiltrated by a large number of mononuclear cells in the challenged animals, the authors speculated that impairment of IGF-I production may render the glioma cells more susceptible to the immune response. Indeed, in a second study the same group was able to demonstrate that subcutaneous injection of IGF-I antisense-transfected glioma cells into rats prevented the formation of both subcutaneous and brain tumors induced by nontransfected cells (Trojan et al., 1993). Most intriguingly, administration of IGF-I antisense-transfected glioma cells was even able to cause regression of already established intracranial glioblastomas when injected ectopically. Although the mechanisms underlying these impressive results are still largely unknown, it appears that a glioma-specific immune response involving CD8$^+$ lymphocytes plays a key role. Trojan and colleagues (1993) have suggested that antisense blocking of IGF-I expression may reverse a phenotype that allows C6 glioma cells to evade the immune system.

ANIMAL MODELS OF GLIOMA INDUCTION

The highest incidence of spontaneous central nervous system tumors of nonhuman mammals is found in brachycephalic dogs. About 60% of these tumors are of neuroectodermal origin (McGrath, 1962). Over the past two decades, several animal models of glioma induction have been developed, using either chemical carcinogens or oncogenic viruses as causative agents. While the former were mainly used in the elucidation of basic mechanisms of tumor evolution in the central nervous system, the latter have become preferred systems in the evaluation of chemotherapeutic agents (Kleihues et al., 1976; Jänisch and Schreiber, 1977). More recently, tumors of the central nervous system have been induced in rodents by transfer of defined oncogenes or combination of oncogenes with or without concomitant carcinogen treatment (Kleihues et al., 1990).

Chemical Neurooncogenesis

The most effective neurooncogenic compounds are simple alkylating agents, particularly nitrosourea derivatives, which cause central nervous system neoplasms in rats after systemic administration (Druckrey et al., 1967). Dialkyl-aryltriazenes (Preussmann et al., 1974) and azo-, azoxy- and hydrazo compounds (Druckrey et al., 1968) are similarly effective. Ethylnitrosourea and related ethylating agents are particularly powerful when administered as a single (pulse) dose transplacentally or shortly after birth (Ivankovic and Druckrey, 1968). With these agents, the susceptibility of the rat central nervous system begins at the 10th prenatal day (E10), increases gradually and reaches its maximum at birth when a single dose is approximately 50 times more effective than in adult rats. After birth, the susceptibility of the central nervous system decreases and reaches that of adult rats at approximately 1 month (Ivankovic and Druckrey, 1968). *N*-Nitrosomethylurea and related methylating agents also induce brain tumors transplacentally, although—due to their higher toxicity—to a lesser extent. These agents produce a high incidence of central nervous system neoplasms when given as multiple small, weekly doses to adult rats. The tumors induced by alkylating agents have been classified as oligodendrogliomas, astrocytomas, ependymomas and mixed gliomas. In addition, transplacental and perinatal exposure to *N*-nitrosoethylurea (ENU) causes the induction of a high incidence of malignant schwannomas of the cranial and peripheral nerves (Wechsler et al., 1969). While the predominant tumor type, oligodendrogliomas, are thought to originate from the white matter, the other gliomas are more likely to originate from the subependymal matrix zone of the lateral ventricles (Schiffer et al., 1978; Burger et al., 1988). Recent immunohistochemical studies have shown that ENU-induced neoplasms previously classified as ependymomas express the neuronal marker synaptophysin and may thus be better termed primitive neuroectodermal tumors with neuronal differentiation (Vaquero et al., 1992).

Malignant transformation by alkylating agents is thought to result from interaction of the ultimate carcinogen, that is, a methyl or ethyl cation, with cellular DNA (Kleihues and Rajewsky, 1984). Of the various resulting base modifications produced, nucleophilic substitution at the extranuclear oxygen atoms of guanine, thymine, and cytosine has by far the greatest mutagenic efficiency and grossly correlates with the carcinogenic potential of the respective carcinogen (Singer and Grunberg, 1993). The major O-alkylated base is O^6-alkylguanine, which, during DNA replication mispairs with deoxythymidine, causing GC → AT transition mutations. O^6-methyl- and O^6-ethylguanine are repaired by the O^6-alkylguanine-DNA alkyltransferase (Pegg, 1990), and this occurs much less efficiently in the brain, that is, the target tissue, in comparison with the liver and other extraneural tissues (Goth and Rajewsky, 1974; Margison and Kleihues, 1975). This was thought to be the mechanism of preferential induction of nervous system tumors, but comparative studies in mice and gerbils showed that their central nervous system is similarly deficient in the repair of O^6-alkylguanines, although ENU and related agents do not have a significant neurooncogenic potential in these species (Kleihues and Rajewsky, 1984). So far, no transformation-associated gene has been identified to play a role in the evolution of ENU-induced central nervous system tumors. In particular, *ras* or *p53* mutations were not found in these neoplasms. In contrast, the genetic basis of ENU-induced schwannomas has been elucidated. These tumors invariably contain a T → A transversion in the transmembrane domain of the *neu* protooncogene (Bargmann et al., 1986; Perantoni et al., 1987).

The usefulness of these model systems for histogenic studies of central neuroectodermal tumors has been severely impaired by the unpredictability in location, number, and histological character of the induced neoplasms. To obviate these difficulties, we have used an organ reconstitution system in which the telencephalic anlage of ENU-primed rat embryos was transplanted into the basal ganglia of congenic recipients. The recipient animals were then challenged with a further dose of the carcinogenic agent (Burger et al., 1988b). Intriguingly, all neoplasms induced within the neural transplants had the typical histopathological features of differentiated oligodendrogliomas. They appeared to develop in a sequence similar to that observed in rats transplacentally exposed to ENU. When animals were allowed to survive for longer periods of time, a tendency to progress toward increased malignancy was observed with increasing histological dedifferentiation and invasion of adjacent brain structures.

Under the conditions of neural grafting, neoplastic transformation is restricted to oligodendrocytes or a precursor cell population already committed to oligodendrocytic differentiation. This is in marked contrast with the variety of neuroectodermal tumors induced by transplacental administration of the carcinogen. It seems, therefore, the ENU-induced brain tumors can arise by two histogenetic pathways: (1) transformation of embryonic matrix cells leading to the development of neoplasms which have retained the multipotent developmental capacity of their cells of origin. These neoplasms often originate

from the persistent subependymal matrix layer and can develop into a variety of histological subtypes, including oligodendrogliomas, astrocytomas, ependymomas, and mixed gliomas; (2) transformation of oligodendrocytes or of precursor cells committed to oligodendrocytic differentiation. This hypothesis is corroborated by the observation that ENU-induced oligodendrogliomas are frequently located in the hemispheric white matter, whereas astrocytic tumors and mixed gliomas tend to arise from the subependymal region (Schiffer, et al., 1978; Lantos and Pilkington, 1979). In fact, the absence of a persisting subependymal matrix layer might well explain the histological uniformity of the observed tumors in telencephalic rat transplants.

Direct Introduction of Oncogenes into Neuroectodermal Cells

In an effort to identify the specific molecular changes responsible for the induction of gliomas, we have exploited the grafting technology outlined above to directly introduce activated oncogenes into neuroectodermal precursor populations *in vivo*. Single-cell suspensions prepared from fetal rat brains were infected with replication-defective retroviral vectors encoding oncogenes and injected into the caudoputamen of adult rats using a stereotaxic frame. Given the frequent finding of activated, overexpressed or deregulated tyrosine kinase oncogenes, we reasoned that it might be interesting to study the consequence of widespread activation of oncogenes belonging to the "intimate" *SRC* family. These oncogenes share various structural and functional characteristics, and many of them are highly expressed in the developing nervous system and in some human and experimental neuroectodermal tumors. The members as yet recognized of this growing family of oncogenes are c-*src*, c-*fyn*, c-*yes*, and c-*yrk*. To deregulate the activity of these tyrosine kinase proteins we exploited a viral oncogene, the middle T antigen of polyomavirus (mT). Although mT lacks an enzymatic activity, it is capable of forming a protein complex with several members of the *src* family and of upregulating their tyrosine kinase activity (Courtneidge and Smith, 1983; Courtneidge, 1987; Kornbluth et al., 1987; Cheng et al., 1988; Kypta et al., 1990).

In rats carrying transplants expressing the polyoma middle T antigen we observed endothelial hemangiomas in the graft, which in 70% of the recipient animals led to fatal cerebral hemorrhage within 13 to 50 days after transplantation (Aguzzi et al., 1991). In the meantime it has been shown that endothelial cells constitute a prime target for the carcinogenic action of mT (Bautch et al., 1987; Williams et al., 1988, 1989; Montesano et al., 1990), and that tumors of other histological type than endothelial probably arise only in situations where neoplastic transformation of the endothelial compartment is blocked (Aguzzi et al., 1990; Rassoulzadegan et al., 1990; Guy et al., 1992). Most recently, we have shown that transformation of endothelial cells is possible even when either one of the kinases, c-*src*, c-*yes* or c-*fyn*, has been inactivated by homologous recombination (J. Thomas et al., 1993; Kiefer et al., 1994). Interestingly, rats that did not die from hemangiomas developed anaplastic gliomas after several months (Figure 69-5). It appears that the rapid induction of hemangiomas is induced by middle T alone, whereas delayed glioma induction requires additional genetic alterations.

As one of the most prominent biochemical effects of mT is the activation of the *SRC* kinase, we studied the consequences of the introduction of an activated moiety of *SRC*, that is, the v-*src* oncogene of the Rous sarcoma virus. Expression of the v-*src* gene caused a 70% incidence of astrocytic and mesenchymal tumors after latency periods of 2–6 months, but no endothelial lesions. It was found by *in situ* hybridization that these oncogenes are expressed in cells belonging to all types represented in the graft. This indicates that cell type-specific transformation is due to differential susceptibility of the respective target cell to the oncogenes, rather than to selective integration or expression of the retroviral construct (Aguzzi et al., 1991).

The highly efficient gene transfer by retroviral vectors into fetal brain transplants provides a challenging experimental strategy to study differentiation and oncogenesis in the central nervous system. In addition to the experiments detailed above, we have exploited this technology to study the action of various nuclear and cytoplasmic oncogenes in cells belonging to the central nervous system (Kleihues, et al., 1990; Brüstle et al., 1992a,b; Wiestler et al., 1992a, 1992b), as summarized in Figure 69-5. This model system may also become useful in assessing the organ-specific transforming activity of specific *p53* mutations observed in spontaneous and inherited human neuroectodermal neoplasms.

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Vector and oncogene	Latency period	Pathological phenotype
LTR *neo* TK Py mT LTR	2 weeks 6-9 months	Hemangiomas Gliomas
LTR *neo* TK v-*src* LTR	2-3 months	Astrocytomas, glioblastomas
LTR *neo* TK k-*fgf* LTR	2-3 months	Vascular proliferations
LTR v-*gagmyc* *neo* LTR	3-4 months	PNET (very rare)
LTR v-Ha-*ras* *neo* LTR	3-4 months	Anaplastic gliomas
LTR v-Ha-*ras* v-*gagmyc* LTR	3-4 weeks	Undiff. neuroectodermal tumors
LTR SV40 LT TK *neo* LTR	2-3 months	PNET (highly efficient)

FIG. 69-5. Cell type-specific neoplastic transformation induced in fetal rat brain transplants through retrovirus-mediated oncogene transfer. PymT is the polyoma virus middle T antigen; v-*src* is the transforming gene of the Rous sarcoma virus; k-*fgf* is the Kaposi sarcoma-related fibroblast growth factor, v-Ha-*ras* and v-*gagmyc* are the activated retroviral forms of the respective cellular oncogenes. [Modified from Aguzzi et al. (1991) and Brüstle et al. (1992a), with permission.]

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