



Neuroglia in the Aging Brain

Edited by
Jean de Vellis

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Neuroglia in the Aging Brain

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Foreword by

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Dedication

for Antonia Vernadakis

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Foreword

As we begin the second millennium and evaluate human achievements, one of the greatest accomplishments of civilization is unquestionably the extraordinary increase of human life expectancy. In prehistoric and early historic times, the average span of life was as short as 25 years. In 1900, a child born in the United States might expect to live only about 50 years, given the mortality conditions of the time. Currently, in most developed countries, life expectancy has risen to around 75 to 80 years. Thus, about half of the remarkable increase in life expectancy has taken place during the 20th century.

Many factors have contributed to this rapid and unprecedented change, among which may be listed not only advances in biomedical sciences (e.g., discovery and use of antibiotics, an understanding of the structure of DNA and the gene), but also improvements in socioeconomic conditions (e.g., higher incomes, better access to medical/health care, better sanitation). One result has been the rapid aging of human populations. Now, greater numbers of individuals are surviving to become centenarians, with ages that approach or even exceed those thought previously to represent the maximal potential life span. Moreover, human life span appears still to continue to lengthen.

Given the rapidly increasing proportion of the aged in the present population, especially those 80 years and older, it is important to understand the physiopathology of aging to be able to improve and prolong functional competence and to prevent or treat the disabilities and diseases of old age. Despite numerous theories of aging at the molecular level [e.g., genetic mutations, DNA damage and faulty repair, expression of genes (gerontogenes, longevity assurance genes) promoting or inhibiting cell proliferation, and role of telomeres], at the cellular level (e.g., wear and tear, oxidative damage), and the organismic level (e.g., disruption of neuro-immuno-endocrine interactions), the cause(s) and nature of the aging process remain elusive.

In complex organisms (mammals and especially humans), the central nervous system, closely related to the immune and endocrine systems, plays a key role in regulating the various stages of the life-span from early development to childhood, adulthood, and old age. Thus, alterations in the nervous system associated with age, such as may occur in neurodegenerative diseases, may impair the capacity of the affected individual to adapt to environmental demands, weaken physiologic competence, and induce death. Aging-associated changes in neural structures and metabolism, synapses and neurotransmission, cell number and regeneration, growth-promoting or inhibiting factors, all play a crucial role in endowing the nervous system with a certain degree of plasticity. Plasticity in this sense is the capacity to show compensatory/adaptive responses on demand (e.g., generated by environmental stimuli/damage). This property, until 20–30 years ago, considered a privilege of the developing nervous system, is now viewed as potentially extended to include the adult and even the old central nervous system.

Indeed, the study of the aging nervous system and, particularly, of neurons, is one of the most active areas of research in the field of gerontology and geriatrics. Less well-known are changes with aging in glial cells and the factors that may modulate aging of these cells. However, it is expected that knowledge of the importance of glial cells in their relation to neuronal metabolism and transport (astrocytes), to myelin formation (oligodendrocytes), and as representative of the immune system (microglia) will become considerably enhanced in the near future. With increasing progress in central nervous system rehabilitation, the restoration of neuronal plasticity after damage caused by trauma, disease, or aging is becoming a reachable reality. Although adult neurons usually do not proliferate, this does not necessarily signify that they have lost the capacity to divide; perhaps inhibitory factors derived from the matrix or even the glial cells prevent adult neurons from manifesting this property. Such inhibition of cell division would ensure neuronal stability desirable for optimal nervous system function. However, under appropriate endogenous influences of their microenvironment, neurons may regenerate, not only in rodents and primates, but also in human brains. Because glial cells with the intercellular matrix and the blood vessels comprise this microenvironment, it may be argued that the study of factors that influence glial function represents a promising, albeit indirect, approach to eventual therapeutic manipulation of neuronal potential for self-renewal. Keeping this important role of neuroglia in mind, *Neuroglia in the Aging Brain* has been organized around six major topics. First to be examined are the cellular and molecular changes that occur with aging, especially in astrocytes, the aging-associated gliosis and its relation to neuronal injury and repair. This is followed by a discussion of neuron–glia intercommunication and of how glial signals may be modified/modulated by neurohormones, hormones, extra- and intracellular metabolism and transport, as well as aging of the blood–brain barrier. The last chapters examine the role of neuroglia, especially that of astrocytes, in the etiology of a number of neurodegenerative diseases and evaluate possible therapeutic interventions, specifically on glial responses, but also, indirectly on neurons.

Planning for this book was envisioned first by Dr. Antonia Vernadakis to whom the book is dedicated. She outlined the major topics to be considered, she enlisted the majority of the contributors and secured a publisher. However, her death in 1998, brought the preparation of the book to a halt and threatened its future completion. The book contributors, well aware of the basic and practical significant contribution of glial development, function and aging to CNS plasticity, were eager to see the book published. Therefore, I know that I express the feelings of all contributors, including my own, when I gratefully acknowledge the willingness of Dr. Jean De Vellis to take over the task of editing the book, which he has done with great expertise. We are also grateful to Elyse O'Grady from Humana Press for her steadfast support of the book publication.

Paola S. Timiras

Preface

The study of the neuroscience and neurobiology of aging continues to be an intensely active area of research. However, most of the research has been conducted on neurons. *Neuroglia in the Aging Brain* reviews the current knowledge of the supporting structure of the nervous system, the neuroglia, in aging.

Neuroglia in the Aging Brain discusses the role of glial cells in normal aging and in pathological aging, i.e., neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, because a great deal of knowledge can be gleaned from the study of these more severe forms of aging. The book is divided into six sections: cellular and molecular changes of aged and reactive astrocytes; neuron–glia intercommunication; neurotrophins, growth factors, and neurohormones in aging and regeneration; metabolic changes; astrocytes and the blood–brain barrier in aging; and astrocytes in neurodegenerative diseases.

It is my hope that all of those scientists engaged in this interesting area of research will find this book to be useful. Many thanks to all of the authors for their fine contributions.

Jean S. de Vellis

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I

CELLULAR AND MOLECULAR CHANGES OF AGED AND REACTIVE ASTROCYTES

Neuromorphological Changes in Neuronal and Neuroglial Populations of the Cerebral Cortex in the Aging Rat

Neurochemical Correlations

**Maria Angeles Peinado, Manuel Martinez, Maria Jesus Ramirez,
Adoracion Quesada, Juan Angel Pedrosa,
Concepcion Iribar, and Jose Maria Peinado**

1. INTRODUCTION

The cerebral cortex is one of the most important anatomic foundations of cognitive and memory functions; both functions are affected by aging, particularly when this process is associated with dementias such as the Alzheimer type (1,2). Nevertheless, available knowledge about the cytoarchitecture and quantitative structural changes in normal brain aging, particularly in the cortex are still scarce and even contradictory (3,4). The heterogeneity of the studies, as well as the fact that the effects of aging differ in cerebral zones, animal models, and individuals, are the main causes of these discrepancies (2,5).

In this chapter we examine the effects of aging on the cerebral cortex from two different but complementary views: The neuromorphological and the neurochemical. The neuromorphological studies reviewed here involve quantitative and cytoarchitectonic analyses of neuronal and neuroglial populations. Neurochemical studies evaluate endogenous levels of cytotoxic amino acids such as glutamate and aspartate, which may act as neurodegenerative agents, as well as determine some aminopeptidase activities involved in neuropeptidergic metabolism, that may affect the free amino acids pool.

2. MACROSCOPIC CHANGES

The first studies of cerebral aging were done by pathologists at the beginning of the twentieth century. They detected morphological differences between sections from young and aging brains, and showed that the nerve cells of aged brains had many deposits of a brown pigment called lipofuscin (6). Today it is known that the senescent human brain weighs an average of 7–8% less, at 65 of age, than a mid-life adult's brain. Magnetic resonance studies suggest that this decrease is greater in white than in gray matter (7), although there is considerable variation between different brain regions (8). In the cerebral cortex the overall reduction in volume is about 5%, with losses close to

10% in the frontal lobe, but only negligible losses in the parietal and occipital lobes (9). Our volumetric studies of the frontal and parietal cortex of the rat indicate stability with aging. No changes with age were detected in any of the different layers (I, II–IV, V, and VI) of these cortical areas (10–13).

3. NEURONAL ATROPHY AND DEATH

Volumetric studies are incomplete if nerve cell populations are not evaluated. In 1984, Haug and coworkers studied 76 human brains and found decreases in volume, stability in the total number of neurons, and therefore increases in neuronal density. These findings were explained as a progressive loss of neuropil but not neuronal loss. The shrinkage of the extracellular space would compensate neuronal swelling and gliosis (14). Our studies in the rat cortex also included quantitative analyses of neuronal density and the total number of neurons; we did not detect significant neuronal losses, although there were some cytomorphometric changes consisting of small but significant decrease in the size of the neuronal nucleus in the frontal cortex (10), as well as in the size of the neuronal soma and nucleus in the parietal cortex (11). These results, in addition to the detection of some dark neuron profiles and gliosis in the aged rats (see below), suggested that aging produced a slight but real neurodegenerative damage in the cerebral cortex, which was particularly evident in rats up to 32-mo-old. This is in agreement with other studies that described neuronal atrophy and the development of age-related neurodegenerative changes. In this sense, a progressive regression of the dendritic tree and neuronal atrophy have been reported in the aging frontal and temporal cortices (14,15). This progressive loss of dendrites leads to partial deafferentation of the neurons (16) and to a decrease in the number of synapses (17–19). The presynaptic bouton's decline in the neocortex of the aging rat seems to be more intense in deeper cortical lamina (20). Although atrophic cells can remain metabolically active during aging (21), the neurodegenerative process may eventually lead to the death of some neurons. However, in contrast to early morphological studies that supported the idea of major age-related neuronal loss (22–24), current studies (25) accept that neuronal death probably occurs only in some specific areas (26) such as the substantia nigra (27), hippocampus (26), and nucleus centralis superior (3). In most regions of the cortex, the total number of neurons, neuronal density, and the percentage of cells per area remain unchanged in aging humans (2,28), monkeys, (29–31) and rodents (10,11,32). In the frontal cortex synaptic density gradually decreases with aging, although synaptic vesicle density remained unchanged (33). These data support the idea of synaptic plasticity in the “functioning” neurons.

We used different histological, quantitative and image analysis techniques (10–13) to evaluate the dynamic changes in neuronal and neuroglial populations in the frontal and parietal cortex during aging. Studies of 10 μm thick histological sections stained with cresyl-fast violet indicated stability in the number of neurons but changes in neurocytomorphometric parameters (10,11). In a separate set of experiments, we used 1 μm sections stained with toluidine blue as well as ultrathin sections. In this new material we detected the appearance of dark neuron profiles (DN) as shown in Figure 1. The results of DN quantification are shown in Figure 2. Only aged rats had a significant increased number of DN in both the frontal and parietal cortex; however, the number of normal neurons (nondark neurons: NDN) decreased with age in comparison to young control animals. In this group the number of DN was imperceptible. Figure 2 also shows that there was no change in the sum of DN plus NDN, total neurons.

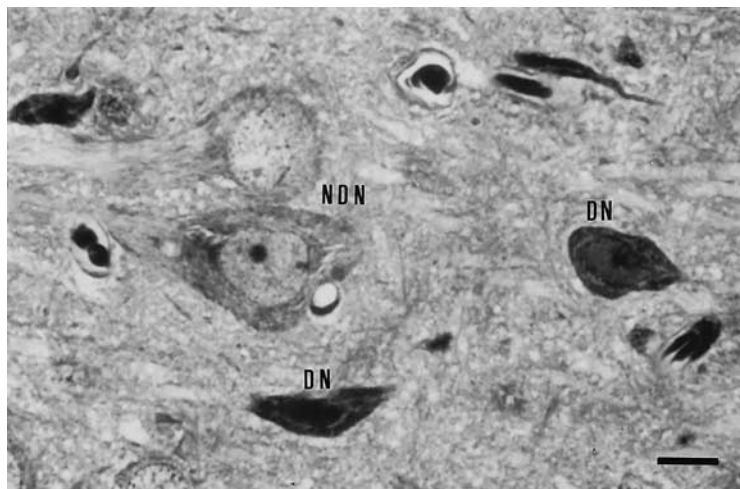


Fig. 1. Toluidine blue-stained semithin section of the parietal cortex of an aged rat showing several DN next to normal NDN (Bar: 10 μ m).

In addition, we analyzed cytomorphometric variables, such as mean neuronal soma and nucleus areas, in a significant sample of DN and NDN in the frontal cortex. Samples were obtained by proportional affixation in different strata throughout the different cortical layers in the frontal and parietal cortices of aged and young rats (12). Figure 3 shows that soma and nucleus area clearly decrease in DN. Considering the scarce number of DN in young rats, cytomorphometric evaluation of the total neuronal population (Fig. 4) (DN plus NDN = TN), indicated only a small decrease in soma and nucleus areas in aged animals as was shown in earlier work (10,11).

DN are characterized by their high affinity for basic stains, their soma, nucleus, and nucleolus are strongly shrunk (34–36). Ultrastructurally they showed microvacuolar degeneration (37), lipofuscin accumulation (38), disaggregated polyribosome and disorganized endoplasmic reticulum and mitochondria (37). Their plasma membrane is irregular and is surrounded by many glial processes (34).

Dark neurons have been well characterized in ischemia (39), glucose deprivation (40), injury (34), transitory breakdown of the blood-brain barrier (41), poisoning with toxins such as kainic acid (42), and elevated atmospheric pressure (43). To explain the formation of these neurons Gallyas et al. (35) proposed a common generative mechanism independently of the cause: They suggested that energy deregulation might affect cytoskeletal neurofilaments (34,43,36).

The detection of DN profiles in histological sections has been interpreted as a sign of neuronal atrophy, probably representing a degenerative step previous to cell death, which occurs later by necrotic or apoptotic mechanisms. Necrosis is the main consequence of mechanical, ischemic or toxic lesions, whereas apoptosis is a type of programmed cell death which normally occurs during development. In addition, necrosis involves an inflammatory response which does not occur in apoptosis. Recent studies describe apoptosis as a common mechanism of cell death in aging, in which intrinsic signals (cytokines, neurotransmitters, growth factors, and so on) as well as extrinsic (mediated by free radicals) signals would participate (44,45). Regardless of the under-

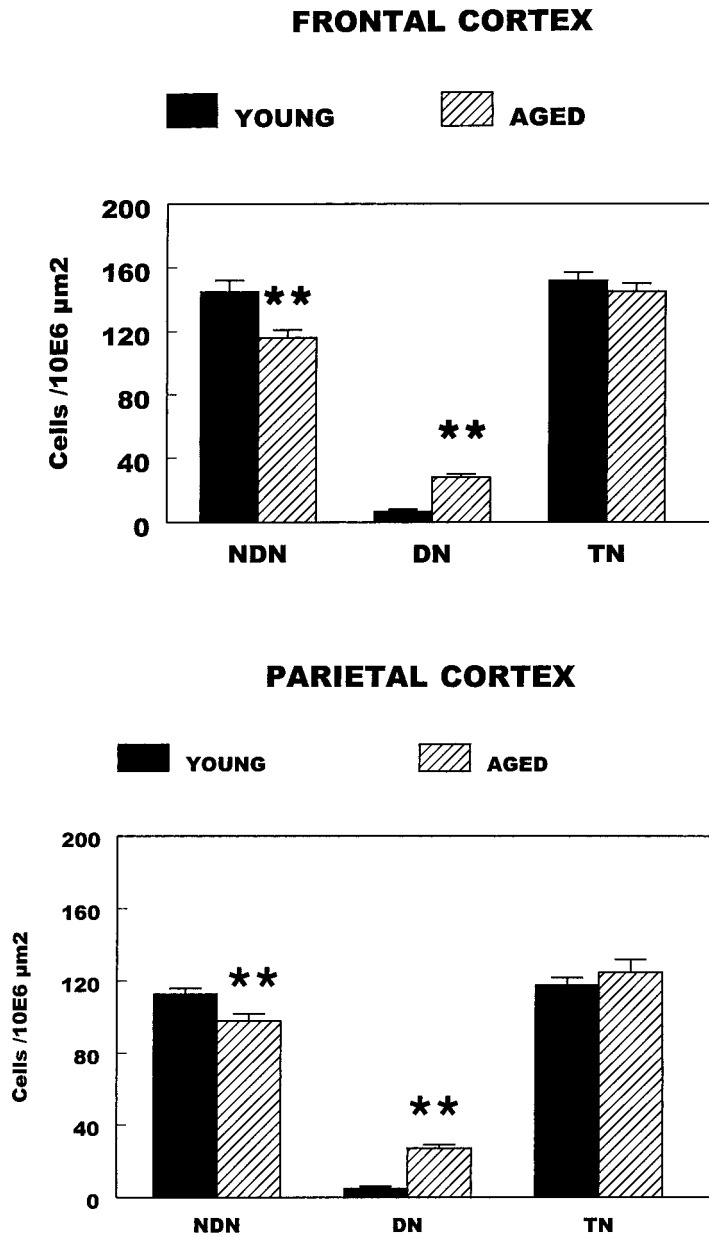


Fig. 2. Neuronal density (mean \pm SEM in $10^6 \mu\text{m}^2$ of nervous tissue) of NDN, DN and total neurons (TN) quantified in the frontal and parietal cortex of young and aged rats. (Frontal young: $n=271$; Frontal aged: $n = 261$; Parietal young: $n = 246$; Parietal aged: $n = 225$.) (For details of the quantitative method see (13).)

lying mechanisms of cell death, we speculate that during aging, DN represent senescent neurons. In fact, atrophic neurons have been described in early aging studies as cells that showed decreases in the size of the pericaryon, nucleus, nucleolus, and dendritic tree (46,47). This general shrinkage may reflect loss of the ability to maintain homeostasis, with gross dehydration and consequently an increased affinity for stains.

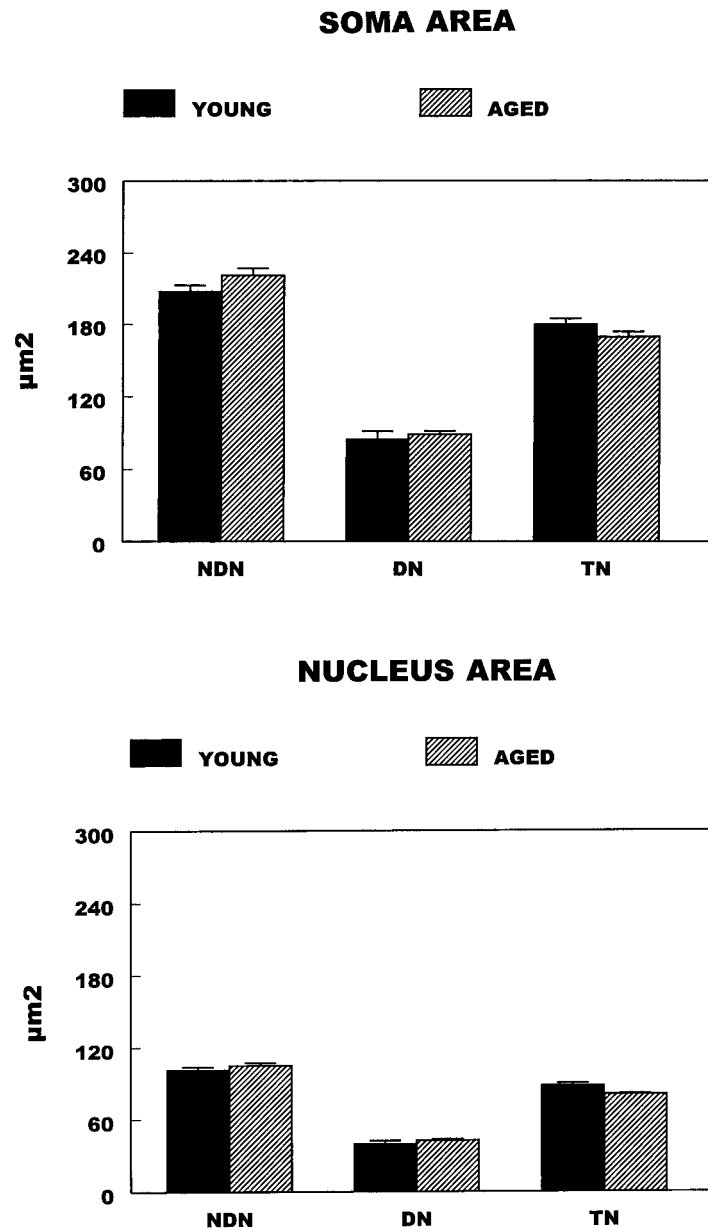


Fig. 3. Neuronal soma and nucleus areas (mean \pm SEM expressed in μm^2) of NDN and DN in the frontal cortex of young and aged rats. (NDN young: $n = 720$; NDN aged: $n = 600$; DN young: $n = 30$; DN aged: $n = 150$.) (For details of the cytomorphometric method see [10,11,13].)

4. REACTIVE GLIOSIS

An other important issue in aging is the behavior of the glial population. Most studies report increases in glial density in parallel with neuronal degeneration (48–50). In morphological studies we detected gliosis in both the frontal and parietal cortex (10–13). Moreover, when we quantified astrocytes and oligodendrocytes-plus-microglial

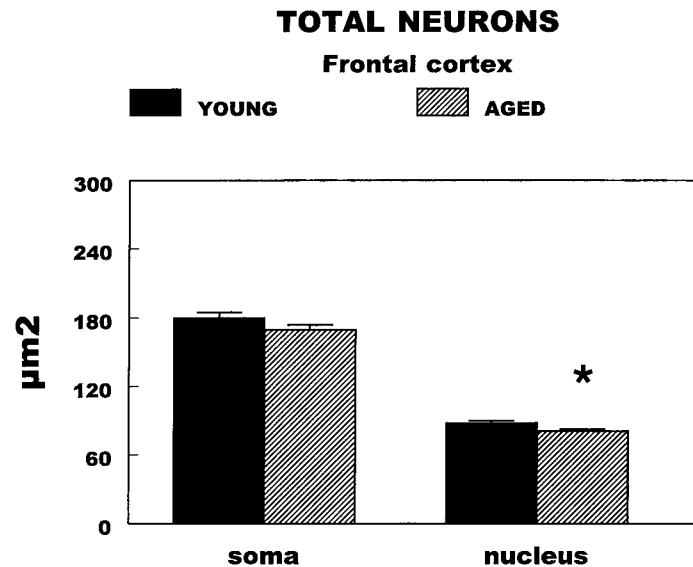


Fig. 4. Soma and nucleus areas (mean \pm SEM expressed in μm^2) of the total neuronal population in the frontal cortex of young and aged rats. (Young: $n = 750$; Aged: $n = 750$.) (For details of the cytomorphometric method see [10,11,13].)

cells, we found the same results in the frontal and parietal cortex: Only astrocytes showed significant age-related increases (Fig. 5). The increases in astrocyte led to hyperplasia and hypertrophy, as shown by the immunodetection of glial fibrillar acidic protein (GFAP) (Fig. 6). These results agree with those of similar studies in humans (51) and rats (52–54).

The changes in glial population may correlate with increases in glucose uptake (55) as well as increases in the activity of enzymes such as glutamine synthetase (56–58) marker of glial function.

Astrocytes may protect neurons (59). These glial cells act as buffers of ions released by neurons during electrical activity (60), inactivate amino acid neurotransmitters (58,61), store glycogen (62) and may release neurotrophic factors that support neuronal function and survival (63,64). Thus, astrocyte proliferation could be interpreted as a consequence of an increasing need for neuronal protection in the aging brain.

We have found that the number of oligodendrocytes plus microglial cells, remain stable with age in the different cortical layers and in the total thickness of the cortex (Fig. 5). In contrast to our results, studies in monkeys (30,65) showed an increase with age in oligodendrocytes and microglia, but not in astrocytes. Other studies, however, have reported no change in the microglial population with age in the mouse brain (66). In addition, Ogura et al. (67), using OX-42 and OX-6 antibodies, also failed to observe changes in the number of microglia, although aging induced their transformation into reactive cells. This was also found by Perry et al. (68), who detected MCH-II antigen in some microglial cells in the brains of aged rodents.

At the ultrastructural level, glial population also undergoes changes with age. These changes include the appearance of foamy inclusions and osmiophilic materials in all glial cell types (30,69). Similarly, some types of abnormal argyrophilic structures or

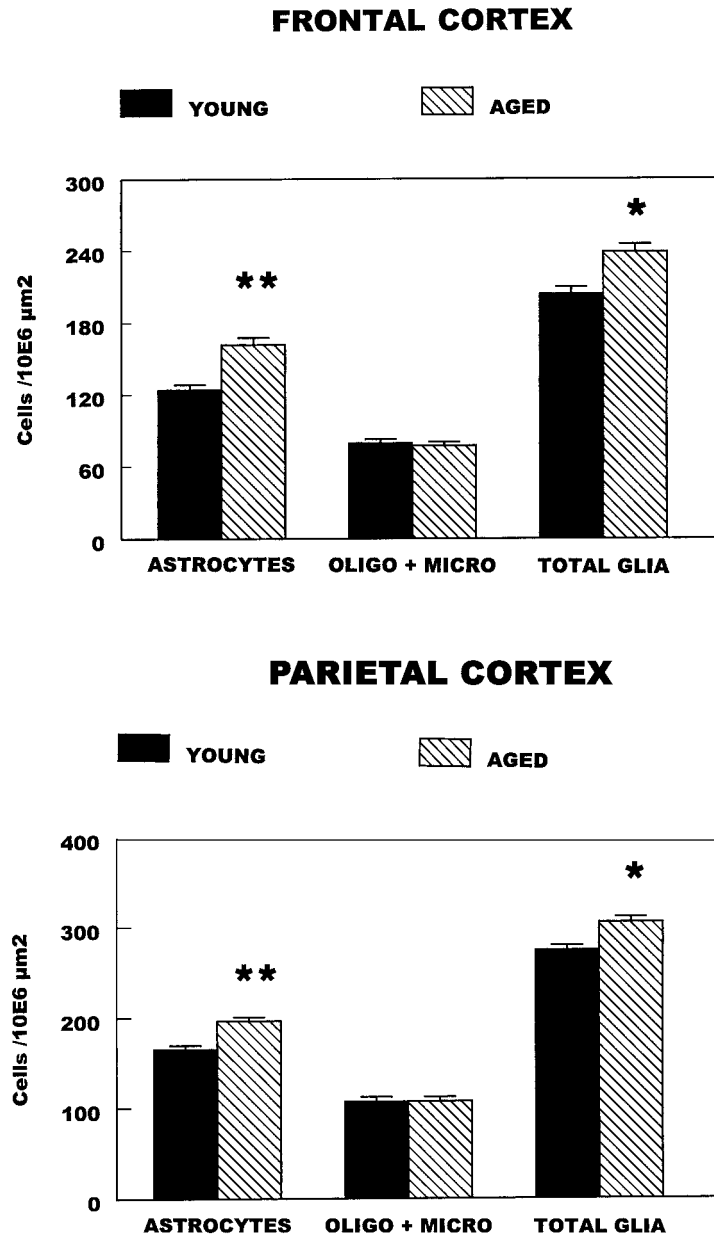


Fig. 5. Density (mean \pm SEM in $10^6 \mu\text{m}^2$ of nervous tissue) of astrocytes, oligodendrocytes plus microglia and total glial cells quantified in the frontal and parietal cortex of young and aged rats. (Frontal young: $n = 500$; Frontal aged: $n = 500$; Parietal young: $n = 300$; Parietal aged: $n = 300$.) (For details of the quantitative method see [13].)

inclusions have been recognized in senescent astrocytes and oligodendroglial cells; immunocytochemical techniques have revealed that phosphorylated *tau*, an abnormal protein located in neurofibrillary tangles, is also present in these cells, although the precise significance of this is still unknown (70).

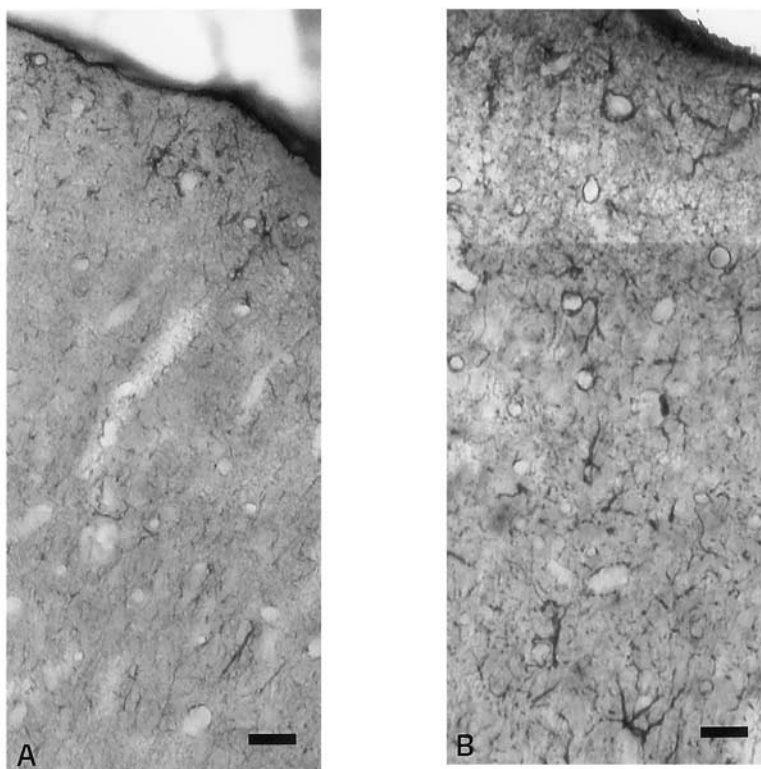


Fig. 6. Immunostaining for GFAP in sections of the frontal cortex of young (A) and aged (B) rats. Note the increase in immunoreaction in the aged rat (Bar: 20 μ m).

5. NEUROCHEMICAL FINDING

All the neuromorphological changes described in aging cells and organism should be interpreted as the consequence of metabolic alterations, including deficiencies in energy metabolism, (71), the production of free radicals (72,73), and changes in neurotransmitter systems such as the cholinergic (74,75), serotonergic, (76) and the aminoacidergic systems (77,78). In normal aging there is an increase in the release of excitatory amino acids, which accumulate in the extracellular space, where they act as potent toxins. The injection of glutamate produces postsynaptic lesions characterized by neuronal swelling and dendritic and axonal degeneration (77,78).

Neuronal as well as glial compartments actively participate in glutamate metabolism (79). The glutamate release from presynaptic neurons interacts with different receptors that can be classified as ionotropic and metabotropic (80). The N-methyl-D-aspartate receptor (NMDA) is a channel that allows the inward flow of Na^+ and Ca^{2+} . This ionotropic receptor has been implicated in the neuronal plasticity and memory functions which are impaired during aging (81,82).

Neurons and glia show mechanisms for the uptake of glutamate which regulate the extracellular concentration of this neurotransmitter, preventing excessive stimulation of glutamate receptors (78). In glial cells glutamine synthetase converts glutamate to inactive glutamine, which returns to the neuronal terminal where it is transformed into glutamate (61). An excess of extracellular glutamate can overstimulate the neurons and

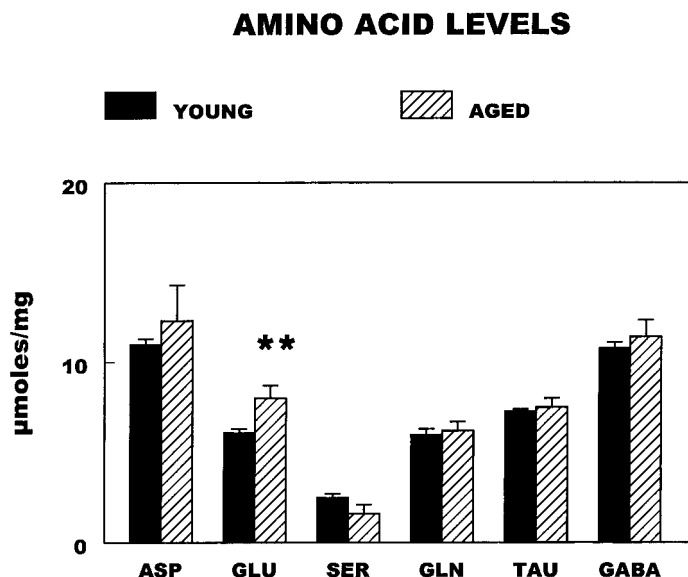


Fig. 7. Levels (mean \pm SEM) of aspartate (Asp), glutamate (Glu), serine (Ser), glycine (Gly), taurine (Tau) and γ -aminobutyric acid (GABA) obtained in the frontal cortex of young and aged rats. Results are expressed as μ moles/mg of nervous tissue ($n = 14$; ** $p < 0.01$).

lead to neurotoxicity. Two mechanisms for these neurotoxic effects have been described. Permanent activation of NMDA produces short-term osmotic damage due to influx of ions/water into the cell, being characterized by neuronal swelling (77,78,83). The long-term mechanism is mainly based on Ca^{2+} permeability. The increase in cytosolic Ca^{2+} activates kinases, proteases and lipases and also generates free radicals. All of these activities can cause neuronal damage (77,78).

We evaluated the levels of aspartate, glutamate, γ -amino-butyric acid (GABA), serine, glycine and taurine in the frontal and parietal cortex of the rat (Fig. 7). The results obtained indicated that only glutamate increased significantly in aged rats. This increase may be at least partly responsible for the neurodegenerative changes observed, and consequently for the memory and cognitive impairments that occur during aging. Furthermore, because astrocytes are to a great extent responsible for glutamate uptake and inactivation, metabolic deregulation of this cytotoxic amino acid activates these cells, which then may proliferate or become hypertrophic, as we found in aged rats.

The uptake mechanism may be inactivated by oxygen free radicals as have been demonstrated in astrocyte cultures (72). In addition, Ca^{2+} through the activation of NMDA receptors, induces nitric oxide (NO) synthesis (81). Considering the role of NO in memory (82), it may be suggested that different mechanisms interact and reinforce the neurodegenerative aging process.

Other mechanisms may also be involved in the deregulation of amino acid metabolism. Proteases participate in neuropeptide activation and inactivation, releasing amino acids and therefore increasing the free amino acid pool. We determined some aminopeptidase activities such as alanine-aminopeptidase (ala-A), α -glutamate-aminopeptidase (α -Glu-Ap), pyro-glutamyl-aminopeptidase (p-Glu-Ap) and aspartyl-aminopeptidase (Asp-Ap). The results obtained (Fig. 8) indicated that only p-Glu-Ap and Asp-Ap were significantly

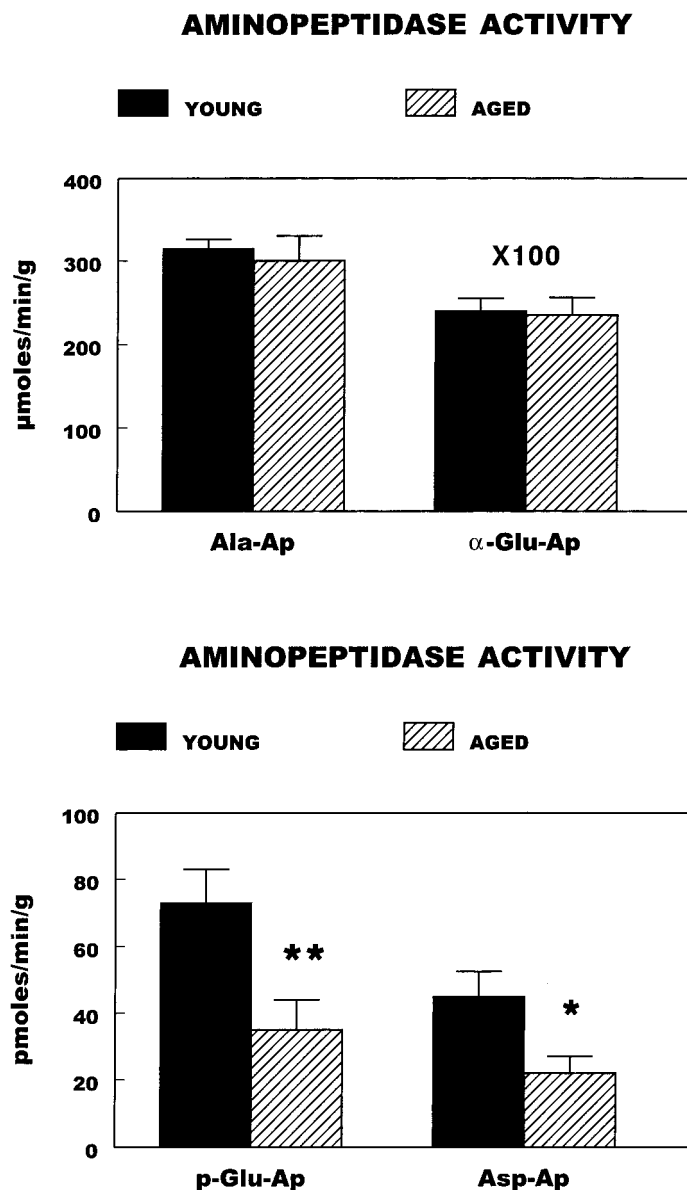


Fig. 8. Aminopeptidase activities obtained for alanine-aminopeptidase (ala-A), α -glutamate-aminopeptidase (α -Glu-Ap), piro-glutamate-aminopeptidase (p-Glu-Ap) and aspartate-aminopeptidase (Asp-Ap), in the frontal cortex of young and aged rats. Results are expressed as $\mu\text{moles/min/g}$ of nervous tissue or pmoles/min/g of nervous tissue. Values of α -Glu-Ap are multiplied by 100 ($n = 14$; * $p < 0.05$; ** $p < 0.01$).

decreased in the cortex of aged rat (84). A generalized decrease in protein degradation and RNA synthesis have been observed with aging in different tissues. In brain, the aged related accumulation of β -amyloid could reflect deficits in protease activity.

In summary, a series of changes occur in the neuronal and glial populations in the frontal and parietal cortex of the aging rat. The aging brain may have a greater suscepti-

bility to functional challenges. Cognitive and memory impairments could be initially due to neuronal dysfunction rather than neuronal loss. This neurodegenerative process would lead finally to neuronal death and reactive gliosis. Changes in amino acid neurotransmitters metabolism could be underlying in this process.

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Diversity in Reactive Astrocytes

Sudarshan K. Malhotra and Theodor K. Shnitka

1. INTRODUCTION

Normal astrocytes in the adult undergo hypertrophy and proliferation and transform into reactive astrocytes following many types of central nervous system (CNS) injury (1–5). This process is termed astrogliosis and may result in the formation of a glial scar.

Morphological studies on astrogliosis by neuroanatomists and pathologists at the beginning of this century focused on the most florid examples encountered in the immediate vicinity of destructive lesions of the CNS, such as lacerations, infarcts, abscesses, and multiple sclerosis plaques (6). Accordingly, the criteria which became standard for defining the reactive state were astrocytic hypertrophy and mild to moderate proliferation, an elaboration of long, thick cytoplasmic processes and an increase in glial filaments composed of glial fibrillary acidic protein (GFAP) (7).

Duchesne et al. (8,9) were the first to draw attention to biochemical diversity in reactive astrocytes in four different models of CNS parenchymal injury. After a hiatus of a decade, there is now growing awareness that reactive astrocytes in different categories of CNS lesions are biochemically heterogeneous, largely as a result of detailed studies of the astroglial reaction in different experimental models of CNS injury (1), combined with the use of a panel of methods to detect GFAP and a number of other conventional and novel “astrocyte-specific” and companion biochemical markers (2,10). From these investigations it is evident that astrocytes do not respond in a stereotypic fashion to all forms of CNS insult, but rather are capable of a variety of types of response, as defined by qualitative, quantitative, temporal and spatial differences in the patterns of molecules which they elaborate in different types of parenchymal injury. This biochemical diversity in reactive astrocytes appears to largely depend on the nature of the CNS injury and the microenvironment of the injury site. Thus, in cellular and molecular terms it is no longer appropriate to hold to a single general definition of astrogliosis.

In this chapter, we consider the likely combinations of “damage signals” and factors in different CNS pathologies that act to induce subtypes of reactive astrocytes. Finally, attention is directed to the future use for mechanistic studies of new and evolving cell culture models of reactive astrogliosis.

2. SUBTYPES OF REACTIVE ASTROCYTES

2.1. Proximal and Distal Reactive Astrocytes in Trauma Models

Proximal reactive astrocytes develop in the immediate vicinity of a destructive lesion of the adult CNS in which there is cellular necrosis and disruption of the blood brain barrier. Distal reactive astrocytes develop at a distance from such a lesion, in a much less disturbed microenvironment (9). The “local astrocytic response” (11) and the “remote astrocytic response” (12) are alternative terms used to describe the foregoing two topographical and intensity-graded patterns of astroglial reactivity.

Proximal reactive astrocytes are the subtype which has been studied in the greatest detail (2–4). Stab wounds of the rat cerebrum provide a convenient and reproducible model system for their induction (1,13). The center of the lesion is necrotic and hemorrhagic. Astrocytes become reactive and undergo hypertrophy within 3 h postinjury in a 1 to 2 mm surrounding zone. The astroglial response spreads through the ipsilateral cortex and into subcortical white matter, reaching a peak between 3 and 7 d. The contralateral cerebral hemisphere also may be remotely affected. The margins of the wound are infiltrated by inflammatory mononuclear cells (i.e., lymphocytes, blood-derived macrophages, and intrinsic brain microglial cells). Between 6 and 12 h postinjury, there is a rapid increase in GFAP mRNA in proximal reactive astrocytes which is followed 2 d later by an increase in their GFAP content (11,14,15). Both GFAP mRNA and GFAP decline to near normal levels by 21 d. Between 3 and 6 d postinjury, approx 13% of the proximal reactive astrocytes are the progeny of cells which have divided (16). Thus, the majority of proximal reactive astrocytes arise from normal astrocytes or their precursors in the region. Over a period of several weeks proximal reactive astrocytes participate in the formation of a persistent astroglial scar (anisomorphic gliosis) whereas distal reactive astrocytes revert to normal (isomorphic gliosis) (6,17). Allowing for differences in the design and execution of experiments, comparable findings to those obtained with cerebral stab wounds have been observed following a cryogenic injury, focal X-irradiation, laser-irradiation, and focal ischemia (2).

Spinal cord transection in adult rats produces an astroglial reaction which is maximal at 14 d. Concurrently, there is both rostral and caudal spread of the reaction (18). In neonatal rats, however, astrogliosis remains limited to the site of spinal cord injury. Barrett et al., (19) suggested that the remote astrocytic reaction is due to degeneration of the long ascending and descending fiber tracts which are myelinated in the adult but not in the neonate. Gliosis is more severe in the lacerated spinal cord than in the lacerated cerebrum, probably because of the greater amount of initial tissue necrosis at the former site. An immunohistochemical study of lacerated adult rat spinal cord by Predy et al., (20), employing a monoclonal antibody (Mab J1–31) raised against multiple sclerosis plaque tissue by means of hybridoma technology (21), revealed that J1–31 antigen is a more intense marker for proximal reactive astrocytes than GFAP, but is a less intense marker for distal reactive astrocytes than GFAP in the rat spinal cord (22) (Figs. 1 and 2).

Many antigens are either unregulated or are expressed *de novo* in proximal reactive astrocytes. GFAP, an intermediate filament protein found only in astrocytes, has become the classical marker for reactive astrocytes in general, and for proximal reactive astrocytes in particular (7). Other molecules which are nonspecifically increased in

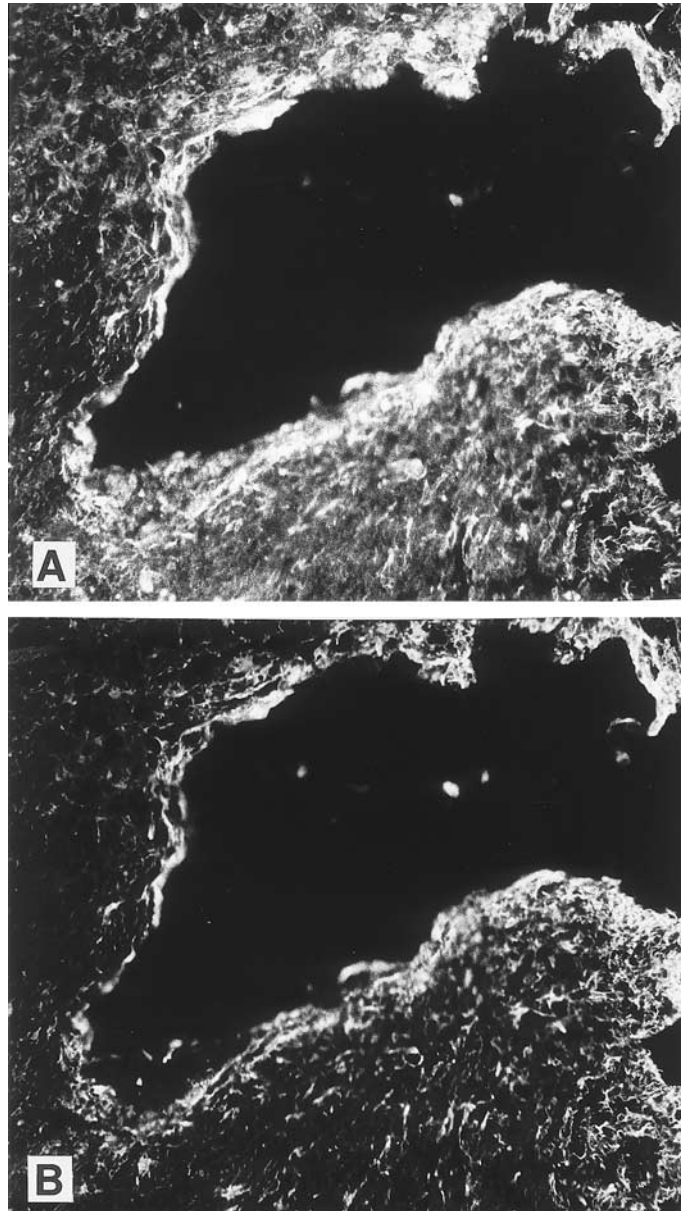


Fig. 1. Sectioned lacerated rat spinal cord showing more intense staining of proximal reactive astrocytes with Mab J1-31 (**A**) than with anti-GFAP (**B**) in the vicinity of the wound. Details of protocols for spinal cord laceration and immunofluorescence staining are given in Predy et al., (20) $\times 106$. (Reproduced with permission from (2).)

proximal reactive astrocytes include S-100 β protein, vimentin; NSE, GS and GAP-43 protein (2,4,5,23).

In recent years, hybridoma technology has fostered the production of some new monoclonal antibodies which recognize specific markers for proximal reactive astrocytes. These include J1-31 antigen (21,22), 6.17 antigen and M22 antigen (5), and

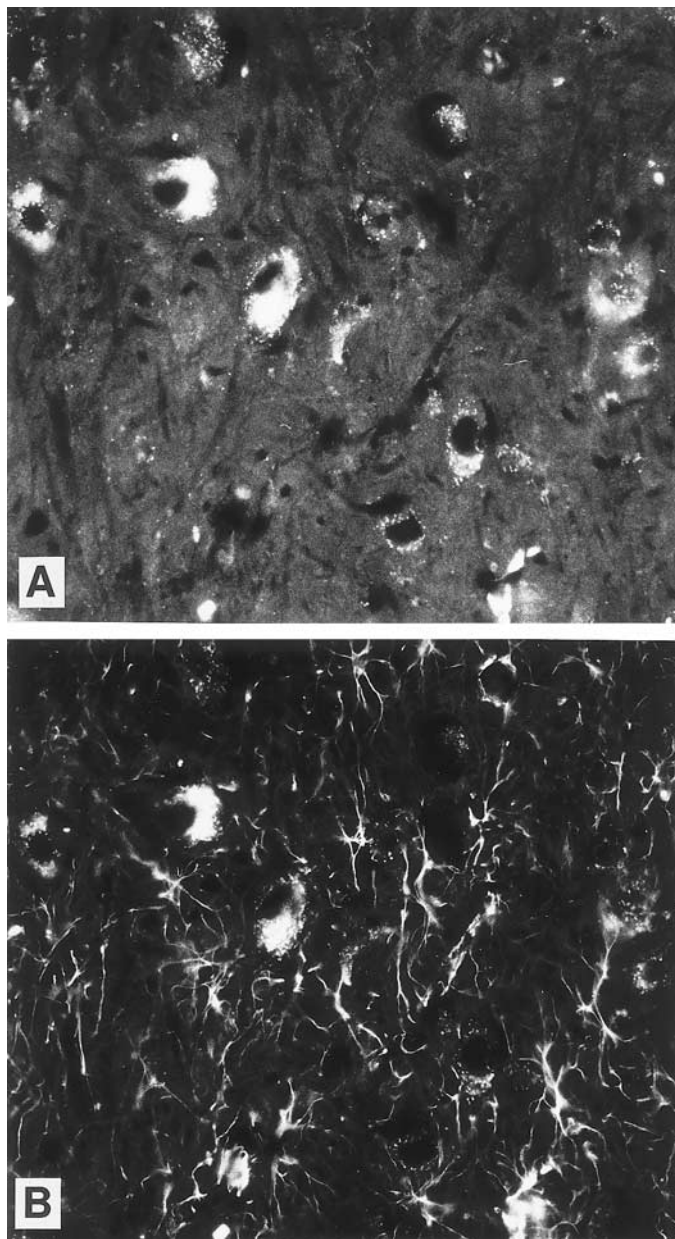


Fig. 2. Sectioned lacerated rat spinal cord in a region at a distance from the wound, showing a lack of astroglial staining with Mab J1-31 (**A**) in contrast to intense staining with anti-GFAP (**B**), in a double-labeled preparation. Autofluorescence is apparent in the soma of neurons in both photographs (arrows). Details of protocols for spinal cord laceration and immunofluorescence staining are given in Predy et al. (20). (Reproduced with permission from (2).)

13All and 01E4 epitopes (24,25). The foregoing require further characterization, however. Table 1 indicates that although proximal and distal reactive astrocytes share some biochemical characteristics in common, they also differ significantly with regard to other characteristics. To date, distal reactive astrocytes have been less thoroughly stud-

Table 1
Comparison of the biochemical characteristics of normal astrocytes, proximal reactive astrocytes and distal reactive astrocytes².

Biochemical characteristics Category ¹	Normal Astrocytes	Proximal Reactive Astrocytes (PRA's)	Distal Reactive Astrocytes (DRA's)
Intermediate filaments	GFAP ↑ GFAP content ↑ Vimentin-neg	GFAP immunoreactivity ↑↑↑ GFAP content ↑↑ Vimentin ↑ (10% of PRA's)	GFAP immunoreactivity ↑↑ GFAP content ↑ Vimentin ±
Gene expression	GFAP mRNA±	GFAP mRNA ↑↑	GFAP mRNA ↑↑
Enzymes	Oxidoreductive enzyme activities: succinate, glucose-6-phosphate, lactate and glutamate dehydrogenases are at normal levels	Oxidoreductive enzyme activities: succinate, glucose-6-phosphate ↑ and glutamate dehydrogenases ↑ Enzymes of glycolysis ↑↑↑ Enzymes of the hexose monophosphate shunt (LDH) ↑↑↑	Oxidoreductive enzyme activities: succinate, glucose-6-phosphate, lactate and glutamate dehydrogenases, remain at normal levels
Growth factors	βFGF-neg	βFGF ↑↑	bFGF-neg
Other biologically active proteins	S-100β protein ↑	S-100β protein ↑↑	S-100β protein ↑
Gangliosides	GD ₃ -neg	GD ₃ ↑	GD ₃ -neg
Other marker epitopes	J1-31-neg 6.17-neg M22-neg	J1-31 ↑ 6.17 ↑↑ M22 ↑↑	J1-31-neg 6.17 ↑↑ M22 ↑

¹ The selection of marker molecules shown above is based on the availability of published comparative biochemical data for normal astrocytes and sub-types of reactive astrocytes under consideration.

² For sources of data see reviews by Eddleston and Mucke (21); Malhotra and Shnitka (2); Ridet, et al., (3); Ridet and Privat (4).

ied than proximal reactive astrocytes. Hence the imbalance in available data concerning these two sub-types of reactive astrocytes (4,23).

2.2. Mechanisms of Activation of Proximal Reactive Astrocytes

The induction of proximal reactive astrocytes in the immediate vicinity of a traumatic lesion of the CNS is a highly complex process involving a multiplicity of “damage signals” and factors and severe alterations in local microenvironmental conditions. The details are far from clear, and remain to be fully elucidated. From pathological studies, it is obvious that mechanical trauma produces local destruction of neural and glial cells and their connections, disruption of blood vessels, and an escape of the cellular and fluid components of blood into the injury site. This complex environment contains serum proteins, blood platelets, eicosanoids, biologically active peptides, myelin breakdown products, fibrin split products and purine nucleosides and nucleotides, and so on, which can activate astrocytes and/or function as trophic factors for mononuclear inflammatory cell (2,4,26). Soon after injury, activated astrocytes and mononuclear inflammatory cells (i.e., lymphocytes, blood-derived macrophages and intrinsic brain microglial cells) and activated endothelial cells elaborate many cytokines (such as ciliary neurotrophic factor [CNTF], nerve growth factor [NGF], basic fibroblast growth factor [bFGF], and insulin growth factor-1 [IGF-1]), which act over short distances in an autocrine or paracrine fashion, to produce a broad range of synergistic or antagonistic effects in different cell types (5,27).

Although the intact normal brain is regarded as an immunoprivileged organ, it does contain resident astrocytes and microglia, which when activated produce a number of potent immune molecules, e.g., TNF- α , TGF β , IL-1, IL-6 and IFN- γ (5,27). TNF- α , IFN- γ and IL-6 have multiple effects in the CNS in controlling glial and neuronal activation, proliferation and survival, thus influencing both degenerative and repair processes. Some of the beneficial and detrimental actions induced by TNF- α and IFN- γ are largely mediated by nitric oxide synthase (NOS)-derived NO production (5). Indeed, NO is involved not only in cytotoxic reactions, but also in the survival and differentiation of neurons. In general, high concentrations are neuroprotective. TNF- α and IFN- γ also upregulate the expression of ICAM-VCAM surface adhesion molecules by astrocytes and brain endothelial cells, thereby favoring astroglial migration, adhesion and anchoring, and neuronal differentiation, during the repair of CNS damage and commencing astrogliosis (27). From the foregoing, it is clear that bidirectional communication exists between resident parenchymal cells (neurons and astrocytes), and resident and infiltrating cells of the immune system in the CNS. It logically follows that *in vitro* studies require *in vivo* confirmation, because indirect synergistic effects predominate *in vivo*, rather than single direct effects (1). The challenge for the future will be to investigate in detail how different factors interact and transform normal astrocytes into proximal reactive astrocytes *in vivo*. The transcriptional mechanisms that are involved in the activation of astrocytes are still a matter of conjecture. There is abundant evidence from *in vitro* studies to indicate that glial cells receive numerous signals which employ cAMP as the second messenger (28,29). Also, signaling through the protein kinase C pathway may play a major role in mediating the developmental proliferation of astrocytes and their activation in astrogliosis in the adult brain (30).

2.3. Mechanisms of Activation of Distal Reactive Astrocytes

The origin of distal reactive astrocytes in both the ipsilateral and contralateral cerebral hemispheres following a cerebral laceration has been the subject of speculation and experimentation (1,13,31,32). Several possible mechanisms have been suggested to explain the phenomenon:

1. The release and widespread diffusion from the site of injury of cytokines and growth factors induces astrocyte reactivity at a distance (31).
2. Neuronal degeneration at the site of CNS injury causes anterograde and retrograde fibre degeneration and includes remote astroglial response in the corresponding projection territories (12).
3. Proximal reactive astrocytes migrate away from the site of injury to remote regions (33).
4. Injury results in decreased gap junctional activity which may be propagated through the astroglial syncytial network (34).

To specifically address the origin of contralateral (remote) astrogliosis, Moudjian et al., (31) performed a callosotomy in rats which also had received a cerebral stab wound, in order to prevent the migration of astrocytes via the corpus callosum. Cerebral callosotomy also served to transect all associative fibers, and thereby enhance neuronal degeneration in both hemispheres. The results of this spatiotemporal study strongly implicated diffusible substances in the induction of the remote astroglial response, rather than the other possibilities listed above. The lack of major local cell destruction, integrity of the blood-brain barrier, absence of blood components, and the pattern of the microglial/macrophage response in the remote astroglial reaction may be additional determinants of its special characteristics.

2.4. Reactive Astrocytes in Axotomy Models

Traumatic injury to a peripheral motor or sensory nerve or the sectioning of a CNS fiber tract (axotomy), induces in the corresponding projection territory, a series of structural and metabolic changes in neuronal cell bodies, dendrites and presynaptic terminals (axon reaction), which are rapidly followed by an astrocytic response (isomorphic gliosis) in the immediate vicinity (35). Details are provided in published descriptions of the axotomy reaction after transection of various motor nerves in the rat (36–41).

After facial nerve axotomy, protoplasmic astrocytes in the facial nucleus undergo hypertrophy and transform into fibrous astrocytes. Increased GFAP synthesis is detectable at 24 h and peaks by 3 d (40). In these motor nerve axotomy models, reactive astrocytes do not proliferate (39), or express vimentin (40), or immunostain with Mab J1–31, (22, see Table 2 and Fig. 3). In the latter context, they resemble the distal reactive astrocytes observed in lacerated rat spinal cord (22).

Until recently, the widely held view has been that “damage signals” emanating from axotomized neurons are responsible for the reactive changes that are observed in neighboring astrocytes. Candidates for the intercellular signaling process have included neurotransmitters (for which astroglial cells possess receptors), or ions particularly K^+ , which is released by active neurons and is taken up by astrocytes (42). Excitatory amino acids (particularly glutamate) may also play a role, because excitotoxicity has been implicated in a number of acute and chronic neurological diseases (43). Also,

Table 2
Comparison of proximal reactive astrocytes induced by cerebral laceration vs reactive astrocytes induced by axotomy

Subtype of reactive astrocyte	Proximal reactive astrocyte	Axotomy-induced reactive astrocyte	References
Model system	Cerebral laceration	Axotomy	
Pattern of glial scar	Anisomorphic gliosis	Isomorphic gliosis	(17,35)
Blood brain barrier	Disrupted	Intact	(71)
Hyaluronate binding protein	Negative	Positive	(72)
Axonal growth	Permissive	Nonpermissive	(72)
Microglial proliferation	Immediate	Delayed	(17)
Astroglial proliferation	Yes	No	(38)
Vimentin	In about 10% of reactive astrocytes	Negative	(16,40)
J1-31 antigen	Postive	Negative	(22)

Reproduced with permission from (2).

reactive changes in astroglia may be related to the synaptic reorganization that follows neuronal injury (42).

However, evidence presented by Svensson et al., (44), strongly suggests that the activation of astrocytes following hypoglossal nerve transection is mediated by factors (such as IL-1) released by reactive microglial cells. Their experimental approach was to block the usual axotomy-induced proliferation of indigenous microglial cells in rat brain by the intraventricular infusion of cytosine-arabioside (ARA-c) (44). Subsequently, astrocytes in the projection territory of the axotomized hypoglossal nerve failed to show expected increases in GFAP and GFAP mRNA, leading to the conclusion that axotomy-induced astrogliosis is mediated indirectly via the microglial reaction.

2.5. Reactive Astrocytes in Cortical Epileptogenic Foci

Temporal lobe seizures are the most common type of active epilepsy in adults, comprising about 40% of all cases (45). A broad range of pathological lesions have been identified in the temporal lobe in association with intractable complex partial seizures, including mild to severe gliosis (Ammon's horn sclerosis), malformation (mild cortical dysplasia, microdysgenesis, tuberous sclerosis, or angiomatous malformations), neoplasms (gliomas or mixed tumors of the CNS), and inflammatory scars from infection or infarcts (46-48).

Ammon's horn sclerosis (AHS) is the most common of the foregoing lesions among patients treated surgically for intractable temporal lobe epilepsy. Pathologically, AHS is characterized by atrophy of the hippocampal formation, loss of neurons and gliosis in CA1 and CA4 and in the dentate nucleus (47). Several theories have been proposed to explain the pathogenesis of AHS, including abnormal circuitry in the hippocampal formation due to a developmental error, hypoxic-ischemic damage (due to birth injury or to post-natal trauma), or that epileptic seizures can cause cortical gliosis (47,49). The currently favored view is that seizures provoke the death of neurons as a result of

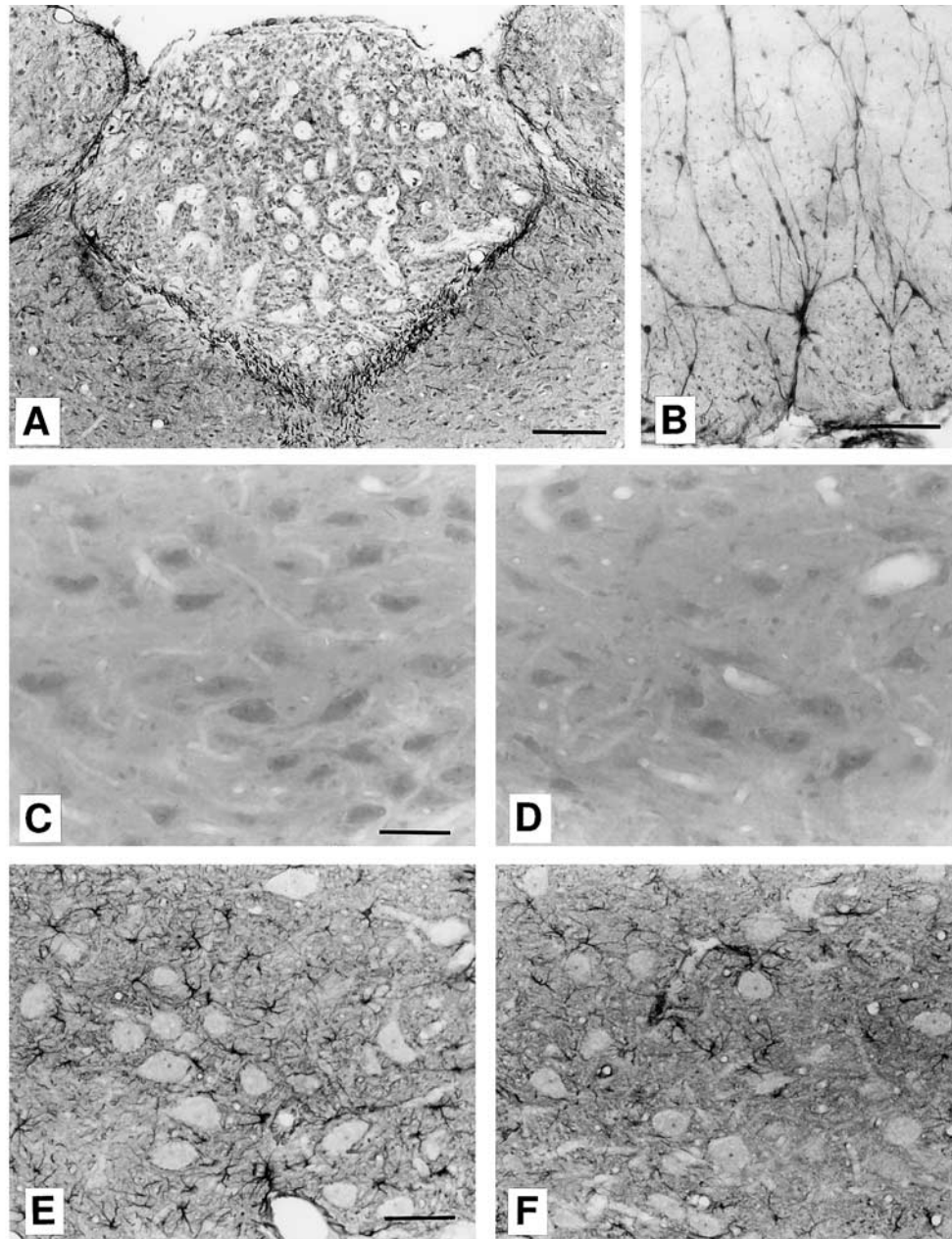


Fig. 3. (A-D) J1-31 immunolabeling of rat brain stem, 1 wk after transection of the right hypoglossal nerve. Immunoreactive processes surround the area postrema and extend toward the central canal (A). Subpial astrocytes and their processes are likewise positive for J1-31 (B). However, no specific immunoreactivity is observed in the right hypoglossal nucleus (C,D). Bar 100 μm (A), 50 μm (B-D). (E-F) GFAP immunolabeling in the right hypoglossal nucleus 1 week after transection of the right hypoglossal nerve. Note the increased intensity of immunoreactivity on the side of the operation (E,F). Bar 50 μm . (Reproduced with permission from (22).)

excitotoxicity. Over-stimulation of neurons occurs when there is excessive activation of glutamate receptors (45). According to this hypothesis, gliosis is a secondary response to the death of neurons. Also, neuronal cell death may be followed by the formation of new synaptic connections with abnormal hyperexcitability.

Steward et al. (42) have shown experimentally that when mild electrical stimulation of the rat hippocampus was employed to elicit neuronal activity and acute-onset seizures, GFAP and mRNA levels increased rapidly and dramatically at the sites of stimulation, as well as in areas that were synaptically activated by the seizures. These early-stage increases in GFAP mRNA did not appear to be related to the presence of necrotic neurons. Candidates for signaling mechanisms included an upregulation of several "immediate early genes" (such as c-fos and c-jun), and an increased release by active neurons of glutamate and K^+ (50).

Experimental models of chronic focal epilepsy have involved the intracortical or topical cortical administration of alumina (51), iron (52) or cobalt (8,53). In relation to the topic of biochemical diversity among reactive astrocytes, Brotchi et al. (54) applied the term "activated astrocytes" to a subset of reactive astrocytes in human epileptogenic cortex and in cobalt-treated rat cortex, which display elevated levels of glutamate dehydrogenase (GS), glucose-6-phosphate dehydrogenase, and lactate dehydrogenase. Glucose 6-phosphate dehydrogenase was the first enzyme to rise and the last to decline to normal (9).

Hamberger et al. (48) have monitored the levels of neuron specific enolase (NSE), two glial cell specific proteins (S-100 β protein and GFAP), and CNAM neural cell adhesion molecule) in surgically excised specimens of human epileptogenic cortex. Gliosis varied from mild to severe. However, no correlation was found between the GFAP content and the duration of focal epilepsy. There was only a 30–40% increase above normal of S-100 β protein. The NSE values were close to normal, which correlated with the observation that the concentration of neurons in the cortex of most patients remains relatively unaffected, even after years of seizures.

2.6. Regional Differences in Resident Normal Astrocytes also May Influence the Development of Sub-Types of Reactive Astrocytes

Controversy exists as to whether the region-specific properties of normal astroglial cells are the result of intrinsic coded information within the glial cell, or whether neurons in different brain regions induce uncommitted astroglial cells to express specific proteins in support of a particular type of neuron-glial interaction (55,56).

In different regions of the intact rat brain, not all normal astrocytes are GFAP-positive (57); also there are large quantitative differences in glutamate dehydrogenase (GS) activity, and S100 β protein in astrocytes in different brain regions (58,59).

Reviews by Hatten et al. (56) and Norton et al. (1) give credence to the suggestion that in certain instances phenotypic diversity in reactive astrocytes may in part be related to the diversity of normal astrocytes in the region. We previously collected together some reports of experiments by others which could fit this pattern of reactivity (2). Two examples suffice here. Alonso and Privat (60) studied the fine structural organization and immunostaining characteristics of reactive astrocytes in glial scars produced in adult rats by two surgical stab wounds in two close-by locations of the same

brain area. With a lesion of the ventral hypothalamus, axonal regeneration occurred and was associated with GFAP-, vimentin-, laminin-, and PSA-NCAM-positive reactive astrocytes. On the other hand, in the dorsal hypothalamus, the same type of lesion resulted in a nonpermissive scar, which exhibited only slight PSA-NCAM and laminin staining. Electron microscopy disclosed that the nonpermissive scar in the dorsal hypothalamus contained significantly more gap junctions than the permissive gliotic scar in the ventral hypothalamus (4).

Another example of regional diversity in reactive astrocytes is described by Fernaud-Espinosa, et al. (17) in a study on the differential activation of microglia and astrocytes in aniso- and isomorphic glial scars. Microglial and macroglial responses were compared after two different types of brain damage in two distinct regions in rat brain (i.e., the cerebral cortex and the hippocampus). Each site was subjected to trauma resulting in anisomorphic gliosis. The microglial response seemed to be only linked to the type of lesion inflicted on the CNS, whereas the astrocytic response also appeared to depend on the region of the brain that was damaged. Reactive astrocytes in the hippocampus expressed β -amyloid precursor protein (β -APP) immunoreactivity after both aniso- and isomorphic gliosis, but this marker protein was not evident in reactive astrocytes in the cerebral cortex, under the same experimental conditions.

2.7. Models of Reactive Astroglial In Vitro

The complexity of the cellular and molecular events occurring in animal models of brain injury has prompted efforts to develop convenient, reliable model systems using astroglial cell cultures, for controlled experiments on astroglial *in vitro*. Wu and Schwartz (25) have reviewed the current range of cell culture models which are available to biochemically characterize reactive astrocytes, i.e., primary cultures of neonatal astrocytes, co-cultures of astrocytes with either neurons or microglia, and organ cultures. Each of the foregoing addresses a different set of questions. The *in vitro* models if employed as a panel, may help to identify the various patterns of “damage signals” and factors responsible for the biochemical diversity observed in reactive astrocytes *in vivo* in different categories of CNS lesions.

Yu et al. (61,62) have described a “mechanical injury model of reactive gliosis *in vitro*” evoked by scratching with a plastic pipet tip, confluent primary cultures of astrocytes prepared from the cerebral cortex of 7- to 10-d-old rat pups. Injured astrocytes along the scratch track started to swell within 6 h of injury, and between 1 and 3 d displayed stellation, an increased content of GFAP, and larger and more organized assemblies of cytoplasmic filaments.

Eng et al. (63) quantitated the changes in gene expression which occurred after a scratch wound of cultured rat astrocytes; *c-fos* mRNA increased 30-fold and heat shock protein (Hsp) mRNA increased four-fold within 60 min. Both returned to low levels by 12 h postinjury. The early genes which are activated may influence cell division, protect against further injury, and induce later genes (such as metallothionein) through the induction of transcription factors.

Two key nuclear signal transduction mechanisms in astroglia appear to be the basis for a flexible genomic switch which allows extracellular signals to change genetic programs and protein expression patterns:

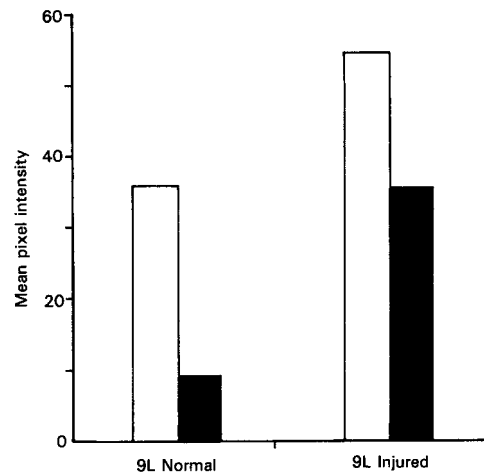


Fig. 4. Astrogliosis in vitro in 9L rat glioma cells induced by mechanical injury (“scratch wound” model). Histogram comparing the relative intensities of the fluorescent signals from double-labeled images for GFAP (solid columns) and J1-31 antigen (open columns) of normal and mechanically injured 9L cells. (Reproduced with permission from (65).)

1. Specific transmitter receptor stimulation by extracellular signals can produce a highly integrated nuclear third messenger (NTM) response with coordinated assembly at the level of NTM transcription and translation;
2. Multiple signals can dynamically change the expression of specific target genes via negative or positive cooperative interactions among various classes of NTMs at the genomic level (64).

In 1995, Malhotra et al. (65) showed that 9L rat glioma cells grown on coverslips, like primary cultures of astrocytes, undergo astrogliosis when subjected to mechanical trauma from a “scratch wound.” Cells along the scratch track display mild hypertrophy and increases in GFAP and J1-31 antigen immunoreactivities, from trace to moderate levels in the case of GFAP, and from moderate to high levels in the case of the J1-31 antigen (Fig. 4). Reaction to mechanical injury by the 9L cells occurred without interactions with microglia, neurons or oligodendroglia. In the same report, we reviewed the literature on the origin and characteristics of the 9L rat glioma cell line and listed the advantages of using cultures of 9L cells, rather than primary cultures of astrocytes, for studies on astrogliosis in vitro. Space limitations preclude the presentation of full details here.

9L rat glioma cells (66), and primary cultures of astrocytes from neonatal rat brain (67) also undergo astrogliosis after exposure to low levels of cadmium chloride (a prototype neurotoxicant [68]). Moreover, when cultures of 9L cells were subjected to mechanical injury and then exposed to cadmium chloride, there was a marked increase in the expression of the astrocytic marker, J1-31 antigen. This was due to a summation of stimulatory effects from these two injurious agents. A moderate coordinated response was detected for the expression of the classical marker, GFAP, and cell hypertrophy was only slightly increased over that produced by either injurious agent alone (Fig. 5). The foregoing findings suggest that more than one transcription mechanism

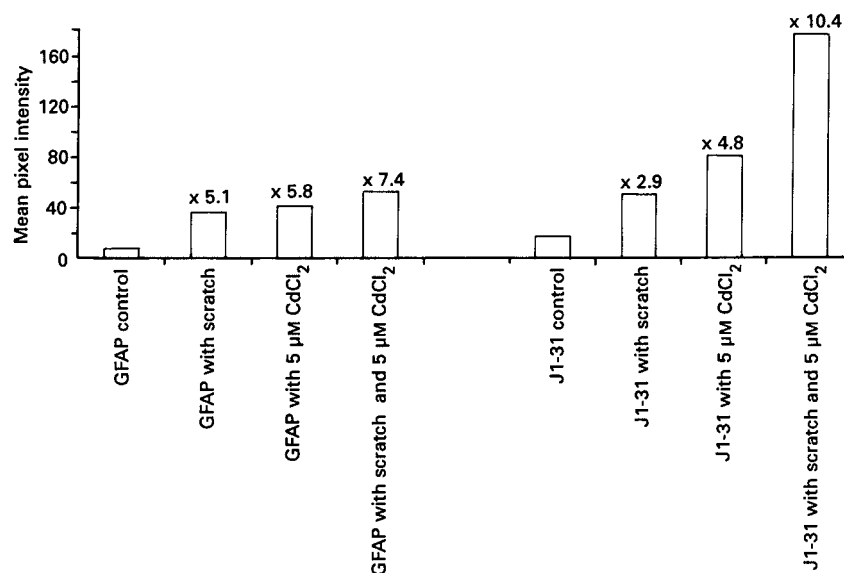


Fig. 5. Coordinated mechanical and chemical injuries upregulate astrogliosis in vitro in 9L rat glioma cells. Histogram comparing the relative intensities of the fluorescence signals from immunolabeled images from 9L cells for GFAP and J1–31 antigen respectively, in normal cells, mechanically injured cells, CdCl₂-chemically injured cells, and in cells with coordinated injuries from both agents. Increases shown above columns. Controls, without scratch or CdCl₂. (Reproduced with permission from (66).)

is involved in the activation of astroglia, and that mechanical and CdCl₂-induced injuries probably respectively affect different receptors and second- and third-messenger pathways. Thus, in a relatively simple in vitro model system, biochemical diversity in reactive astrocytes could be demonstrated by altering the conditions of astroglial cell injury.

3. CONCLUDING REMARKS

Recent reports on a wide range of experimental animal models of CNS injury, combined with the application of immunostaining methods for a considerably expanded panel of conventional and novel astrocyte-specific markers, indicate that reactive astrocytes are capable of graded levels and different types or responses, as defined by the molecular they elaborate in different categories of local pathology (1,2,4). Biochemical heterogeneity and functional diversity are also prominent among other activated types of specialized cells such as microglia (69) and fibroblasts (70). This adaptive plasticity of reactive astrocytes largely appears to be a modulated response to the different micro-environmental conditions, particularly the combinations of immune molecules, cytokines, eicosinoids, serum factors, peptides, nucleotides, and adhesion molecules that exist in different categories of CNS lesions. Regional biochemical differences in the normal astrocytes from which reactive astrocytes arise, as well as local nonglial influences, may be contributory factors in certain brain regions.

Future research, no doubt, will expand on the lines of investigation already established. New and evolving cell culture models of reactive astrogliosis should facilitate

mechanistic studies. The challenge then will be to determine both the common and the diverse signaling mechanism which underlie the conversion of normal astrocytes into their respective reactive subtypes. The foregoing should lead to a better understanding of the special roles played by the biochemical subtypes of reactive astrocytes which are present in different categories of CNS pathology.

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Astrocytic Reaction After Traumatic Brain Injury

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1. INTRODUCTION

Astrocytes and microglia respond to a great variety of lesions in the nervous tissue of the central nervous system (CNS). In this sense, although astroglial cells react in a relatively constant manner, the intensity and time course of the changes are largely dependent upon the type of lesion involved.

The present review fundamentally addresses traumatic lesions affecting the CNS. This type of lesion has been extensively studied from the experimental perspective and constitutes one of the most reliable and easily reproducible models for analyzing the reactive changes taking place in the glial component of nervous tissue.

One of the earliest morphological studies of glial reaction to traumatic injury of the CNS was carried out by Wilson in 1926 (1). This author employed conventional histological techniques (cresyl violet and trichromic stains) to study the trajectory of therapeutic punctions made in two human brains. In this context, a glial reaction was observed, with connective tissue participation in the repair processes. However, the application of metallic impregnation techniques allowed more detailed research of the glial reaction in these lesions, and afforded a clear distinction between cell types such as astrocytes and microglia. In this sense, the classical studies of Penfield et al. were of great interest (2–4). These authors, by producing puncture lesions in the brain, detected a series of cellular changes in the astrocytes that comprised early edema, astrocyte proliferation, fibrous morphological transformation of the astrocytes, astrocyte hypertrophy, and participation of the connective tissue in the glial scar. In addition, the intensity of the glial reaction was related to the amount of necrotic tissue produced.

2. INTERMEDIATE FILAMENT PROTEIN EXPRESSION IN ASTROCYTES

Both glial fibrillary acidic protein (GFAP) and the protein vimentin (VIM) have been described in astrocytes. GFAP is considered to be characteristic of mature astrocytes (5), whereas VIM—initially described in mesenchymal cells—has been detected in radial glia and in mature astrocytes (6,7). Changes in the expression of both glial antigens are thought to occur in the course of normal astroglial development. Thus, in several mammal

species, VIM-GFAP transition takes place in the first weeks of life after birth, as a result of which GFAP and VIM may be temporarily coexpressed by most astrocytes during such transition periods (7–11). Moreover, biochemical studies have revealed changes in the presence of messenger RNA (mRNA) encoding for both GFAP and VIM during this period (12).

Once development has been completed, astrocytes express variable amounts of GFAP. Whereas the concentration of the latter is high in fibrous astrocytes of the white matter (13), it may be very low in certain regions of the brain cortex—where protoplasmic astrocytes (at least in the rat) cannot be detected by the habitual immunohistochemical techniques (14)—with the exception of layer I astrocytes that form the *glia limitans*, which are clearly GFAP-positive. However, astrocytes in special locations such as the cerebellum, retina, optic nerve, and major tracts of white matter continue to coexpress GFAP and VIM even in the adult animal (7,15–17). In vitro immunoelectron microscopic studies of GFAP and VIM location by Abd-El-Basset et al. (18) have shown that in both astrocytes and their precursors, VIM and GFAP copolymerize in the same individual intermediate filament; as a result, the GFAP/VIM ratio present in these intermediate filaments reflects the degree of differentiation and functional status of these cells.

Gliosis is the typical astroglial response to CNS lesions. The term encompasses two phenomena: Astroglial proliferation and hypertrophy (14). Astroglial hypertrophy (proliferation will be addressed in the corresponding section below) is the most important and easily detectable characteristic of reactive astrocytes. It may be demonstrated by argentic impregnation techniques (Cajal's gold sublimate, for example), though at present the immunohistochemical demonstration of increased GFAP (and occasionally VIM) expression is clearly the most reliable parameter.

2.1. Time Course

The early character of astrocyte reaction seems to be a constant finding in traumatic damage of the CNS—regardless of the lesion model employed. Thus, in experimental cerebral wounds, increased GFAP immunopositivity becomes detectable a few hours to two days after producing the lesion—with a maximum intensity peak that rarely exceeds 7 d (18a–27). In relation to this phenomenon, the findings have been similar to those reported for other experimental models of traumatic injury of the CNS (28–34).

It is interesting to point out that the early onset of astrocyte reaction (expressed by an increase in GFAP positivity) is very similar to that observed in other types of nervous tissue lesion (35–59). In different types of lesion, the data obtained from biochemical studies—both as regards GFAP quantification (20,36,55,59–62) and GFAP mRNA assay (46,55,60,62,63)—clearly confirm the immunohistochemical findings. Likewise, GFAP mRNA *in situ* hybridization studies (55,64) have shown the astrocytes to be responsible for GFAP synthesis.

Based on electron microscopic studies of the first evolutive stages of experimentally induced cerebral lesions, we have found (nonpublished personal observations) reactive astrocytes to exhibit a manifest edematous appearance, with a very electron-transparent hyaloplasm and very scarce glial filaments (though the latter subsequently increase greatly in number). In this sense, the ultrastructural image is scantily compatible with the intense GFAP immunopositivity detected in light microscopic immunohistochemical studies. Considering that the intermediate filaments are highly dynamic structures (65), a pool of kinetically active disassembled subfilamentous units would be expected to be in

dynamic equilibrium with assembled filaments (66). The surprising increase in astrocyte GFAP immunoreactivity so shortly after injury, and the apparently paradoxical ultrastructural absence of glial filaments, could be accounted for by a rapid increase in GFAP synthesis, which would expand the pool of subfilamentous units and thus increase the immunopositivity of the reactive astrocytes that nevertheless still require a period of time to complete filament assembly.

As seen above, the constant pattern of findings has led to widespread consensus on the rapid establishment of reactive astrocyte changes in highly diverse CNS lesions. However, variable results have been obtained as regards the permanent or transient nature of such changes, depending on the experimental model involved. An added difficulty is the tendency to carry out studies that conclude too early—thus frequently preventing the recording of reliable data on the true duration of the glial reaction.

Cerebral lesions exhibit a gradual decrease in astrocyte reaction over time, reflected by a drop in GFAP immunoreactivity. In this sense, the reaction is seen to have clearly decreased after approximately three weeks (20). After this period of time, only a narrow band of immunopositive astrocytes remains in the cortex surrounding the lesion (22). Biochemical studies have shown the amount of both GFAP (67) and GFAP mRNA (68) to return to values very close to normal after these three weeks. Likewise, in mild contusive brain lesions, immunopositivity practically returns to normal 30 d after injury (28).

The duration of the glial reaction is much more controversial in other types of CNS lesion involving astrocyte response. Thus, in ischemic lesions the reaction is seen to remain stable after three months (49), though it may descend to negligible levels after six months (44). In turn, deafferentation lesions appear to induce a transient astroglial reaction. As an example, deafferentation caused by the injection of a neurotoxic agent (ibotenic acid) induces a glial reaction in the projection areas of the injected zone that disappears 5–6 mo later (45). The astrocytes of a given region undoubtedly respond to lesions in a subcortical nucleus projecting fibers to that region. In this sense, astrocytes have been shown to respond to cholinergic but not to dopaminergic deafferentation (36). Additional examples of transient reactions are the glial response produced in the olfactory tract by sectioning the olfactory nerve—with regression occurring after one month (35)—and the glial reaction in the facial nucleus after compression of the facial nerve (with regression after 40 d) (57).

However, other lesion models appear to induce a permanent glial response. Thus, in Wallerian degeneration of the optic nerve, the astrocyte reaction seems to persist for at least one year and apparently indefinitely (69,70). Likewise, in unilateral sections of the subcommissural fornix, glial response persists for at least one year (56). Toxic lesions (40,57,71) in turn lead to a practically permanent presence of reactive astrocytes.

In other situations, the greater or lesser persistence of the glial reaction is related to the severity of the damage produced. This has been observed following the induction of variable intensity brain ischemia (53), and in toxic lesions (44), where the astrocyte reaction persists after four months in certain areas of the hippocampus and fades in others—in close correlation to the degree of neuronal degeneration produced.

2.1.1. Other Intermediate Filament Proteins in Astrocytes

Although much less frequently than GFAP, VIM has also been used as a reactive astrocyte marker in traumatic lesions of the CNS. In cerebral injuries after 5 d of evolution, our group (19) has detected a reactive astrocyte band measuring 300–350 μm in thickness sur-

rounding the punction trajectory. By applying techniques for the detection of VIM and GFAP, we were able to show that 60% of the reactive astrocytes coexpressed VIM and GFAP—the remaining astrocytes only being positive for GFAP. In no case did we observe simultaneously VIM-positive and GFAP-negative astrocytes. Other authors have also reported the coexpression of VIM and GFAP in reactive astrocytes (50,53). By using this same lesion model, Takamiya et al. (25) have detected reactive astrocytes that transiently express VIM from 2–10 d post-lesion, with a maximum expression peak after 3–5 d. This latter observation has also been reported in other types of acute necrotic lesions (53).

The expression of VIM has also been described in reactive astrocytes in noninvasive traumatic injuries, such as brain contusions (28,31) or spinal cord compression (29). VIM expression has likewise been recorded in ischemic lesions (44,49,52,54), toxic lesions (44), in lesions involving retrograde degeneration (42,59), or in Wallerian degeneration processes of the CNS (50,56).

As pointed out at the start of this study, VIM is regarded as an immature astrocyte marker in the course of astroglial development; consequently, VIM expression by reactive astrocytes could reflect a temporal regression of these cells to immature states. In this sense, Abd-El-Basset et al. (18) have observed in vitro that highly mobile astrocyte precursors (proastroblasts and young astroblasts) possess intermediate filaments exclusively composed of VIM (proastroblasts) or composed of a heteropolymer in which the GFAP/VIM ratio is low (young astroblasts). In contrast, in more mature (and thus less mobile) astroblasts, and in immobile astrocytes, the intermediate filaments are composed of heteropolymers with a high GFAP/VIM ratio. These authors suggest that heteropolymer formation allows the astroglia to regulate the “stiffness” of its intermediate filament network by increasing or decreasing the GFAP/VIM ratio. The reactive astroglia could behave in the same manner, thus responding to different functional situations that may require increased cell mobility.

The idea that reactive astroglia undergoes a certain regression toward more immature states has been supported by new evidence in recent years. Thus, nestin (a type of intermediate filament expressed by neuroepithelial stem cells of the embryonic CNS) (72) is known to be expressed by reactive astrocytes (along with GFAP) in lesions of the CNS (73–75).

In punction-induced cerebral lesions, reactive astrocytes (27) have been found to coexpress GFAP and a certain intermediate filament associated protein (IFAP) found in radial glia and derived elements, but not in the adult CNS.

2.2. Spatial Spread

In experimental cerebral wounds, astrocyte reaction is frequently not limited only to the proximity of the lesion, but extends through the damaged hemisphere and even to the opposite hemisphere (i.e., theoretically unaffected by the traumatism). The degree of homolateral spread and the presence or absence of contralateral involvement seems to depend upon the size of the lesion. Thus, in small cerebral wounds (18,22,23,25), the astroglial reaction is circumscribed to 1 mm of tissue surrounding the lesion, or alternatively extends throughout the ipsilateral cerebral cortex (18,22,25) but without reaching the contralateral hemisphere. A similar situation is observed in percussion-induced contusive brain lesions (33). It is interesting to note that the astroglial reaction in the damaged hemisphere is later in developing with a slower and more persistent spread in subcortical regions than in the cortex (18a,22). In contrast, when the amount of damaged tissue is

greater (24), the reaction likewise extends to the cortex opposite to the side of the lesion, albeit with less intensity. Unpublished observations by our group corroborate this finding. In addition, we have established that VIM is only expressed by the reactive astrocytes immediately adjacent to the puncture trajectory, as a result of which this protein clearly cannot be employed for studying more distant glial responses. In nontraumatic brain lesions that nevertheless involve abundant damaged nervous tissue, as in experimental laser-induced injuries (53), astroglial reaction in the contralateral hemisphere can also be detected. According to these authors, the expression of VIM by the reactive astrocytes is restricted to the areas in proximity to the lesion.

A phenomenon requiring more detailed analysis is the presence of astrocyte reaction in the undamaged hemisphere. According to Moundjian et al. (24), a number of causes may be postulated:

1. Distant tissue diffusion of soluble factors that induce astrocyte activation and which are initially released at the site of the lesion;
2. Wallerian degeneration necrosis of nerve fibers with cortico-cortical projections that traverse the *corpus callosum*, thereby triggering an astroglial reaction contralateral to the lesion that is in turn incremented by the astrocyte reaction produced by deafferentation itself;
3. Migration of reactive astrocytes away from the wound site, through the *corpus callosum*, with secondary colonization of the contralateral hemisphere.

In this sense, it has been shown that transplanted astrocytes are able to migrate (77–82)—an important migration route being the parallel tracts of myelinated nerve fibers. However, native astrocytes do not appear to possess such mobility (83).

In order to evaluate the contribution of each of the mechanisms proposed above in accounting for the spread of astrocyte reaction to the contralateral hemisphere (24), a number of surgical procedures have yielded the following results:

1. Callosotomy alone induces mild gliosis in both hemispheres;
2. Unilateral brain lesion only produces severe ipsilateral and moderate contralateral gliosis;
3. Callosotomy with unilateral brain lesion induces effects similar to those of the second procedure above.

According to these authors, if astrocyte migration through the *corpus callosum* were a factor to be taken into account, then the contralateral gliosis seen in rats subjected to lesion plus callosotomy (which thus prevents migration) would have to be less than that recorded in rats with lesion only. However, no differences are observed between the two experimental groups. On the other hand, if Wallerian degeneration of axons projecting to the contralateral hemisphere—with the resulting deafferentation—were an important consideration in the genesis of contralateral gliosis, then animals belonging to the lesion plus callosotomy group would be expected to develop more contralateral reactive gliosis than the group subjected to lesion only. Not only is this not the case, but callosotomy alone moreover induces less gliosis than the contralateral reaction of the brain lesion alone. As a result, Moundjian et al. (24) clearly favor soluble factors diffusing from the lesion site as the basic mechanism underlying the observed contralateral glial reaction.

An additional factor must be considered in the origin of distant (and even contralateral) astrocyte reactions. In effect, it is well established that astrocytes are interlinked by gap junctions (84). These cells therefore form a type of “astroglial syncytium” throughout the nervous parenchyma. In experimental astroglial reactions, the immuno-

histochemical expression of connexin-43 (a predominant protein in interastrocyte gap junctions) is found to be increased (85,86). This suggests that transformation to reactive astrocytes implies an increase in gap junctions and thus in the efficacy of the “astroglial syncytium.” In this way, certain intracellular signals that trigger gliosis could pass from one cell to another—covering considerable distances in a relatively brief period of time. However, the fact that VIM expression by reactive astrocytes never extends beyond the injury zone could be in conflict with the idea of possible intercellular signals that diffuse across gap junctions to play an important role in the genesis of distant astroglial reaction.

3. ASTROCYTE PROLIFERATION

The use of double labeling techniques (GFAP and thymidine or similar compounds such as bromodeoxyuridine, widely employed in recent years) is essential for studying astrocyte division in CNS lesions. By applying these methods to nervous tissue lesions with scant production of necrotic material and an intact blood-brain barrier, most studies have shown that the astrocytes do not divide (51,87,88). In contrast, deafferentiation lesions do seem to show astrocyte proliferation (89).

In experimental traumatic lesions of the CNS (23,25,90–99), astrocyte proliferation is generally an early and very brief phenomenon that only takes place between 24 h and 8 d after lesion induction. There is no evidence of astrocyte proliferation in later stages of the evolutive course, and the maximum mitotic peak is reached 3–4 d after injury. The labeling index is low and rarely exceeds 10%. Similar results have been obtained in other traumatic lesion models (28,100).

Regional differences in astrocyte proliferation have been detected. Thus, Topp et al. (26) and Garcia-Estrada et al. (91) have found the labeling index to be higher in the cortex than in the hippocampus, whereas Janeczko (93) has reported greater proliferation in the white matter and deep-lying regions of the lesion. Although the proliferative phenomena appear to be limited to the damaged hemisphere (23,25,93), other researchers have found a slight spread of astrocyte division towards the contralateral hemisphere (101,102).

Thus, it seems clear that although many nervous tissue lesions exhibit an important number of GFAP-positive reactive astrocytes, the low cell proliferation figures recorded indicate that most such cells are preexisting GFAP-negative astrocytes that have become GFAP-positive.

4. PHAGOCYTTIC ROLE OF THE ASTROGLIA

Although the microglia/macrophages are the principal cells in charge of eliminating foreign, nocive, or degenerated elements in nervous tissue injuries, evidence suggests that astrocytes also possess a certain phagocytic capacity.

In vitro research has shown that astrocytes in neonatal or early postnatal rats uptake polystyrene spheres (79) within lysosomal structures, where they are retained for several weeks. These cells are also able to engulf yeast cells (103) and latex particles (104). Ronnevi (105,106) has in turn described astrocyte phagocytosis of synaptic buttons in the neonatal cat.

On the other hand, the phagocytosis of degenerative tissue debris by astrocytes has been described under pathological conditions such as nerve fiber injury and degeneration (63,107–116), toxic lesions (76,117,118), experimental allergic encephalitis or traumatic brain injury (64).

However, ultrastructural studies carried out by our group involving experimental cerebral wounds and Wallerian degeneration of the optic nerve have failed to provide evidence of phagocytosis by astrocytes. In the case of Wallerian degeneration of the optic nerve, we have seen (69) an increase in lipid droplets and dense bodies within astrocytes. Although the dense bodies often adopt peculiar morphologies, we have never observed unequivocal images of astrocyte phagocytic activity. We consider that no clear phagocytic role can be attributed to these cells—at least in the experimental setting involved. In contrast, astrocytes may be implicated in the metabolic processing of certain products originating from intense tissue degeneration—a phenomenon that could explain the presence of lipid droplets.

An interesting experimental approach to the problem of a possible phagocytic role for the astroglia involves the *in situ* administration of particulate material—most of which will obviously be engulfed by the microglia/macrophages. A number of authors have applied this experimental design, using different materials. Thus, it has been found that astrocytes in the neonatal rat phagocytose exogenously injected carbon particles (119), and that astrocytes in adult rats are able to capture latex particles (83).

In a recent study in adult rats subjected to the local injection of colloidal carbon in cerebral wounds, Al-Ali and Al-Hussain (120) have observed that astrocytes are able to phagocytose carbon particles. However, this phenomenon was only recorded in astrocytes in brains subjected to two successive injections of colloidal carbon spaced one week apart; no uptake was seen in brains subjected to a single injection. The evident conclusion of this study is that astrocytes only act as phagocytes in the event of “professional” macrophage saturation, i.e., astrocytes would appear to function as a second line of defense.

5. NEWLY-FORMED GLIA LIMITANS

In extensive traumatic lesions of the CNS, meningeal cells of fibroblastic appearance (meningocytes) penetrate deeply within the cavity of the lesion and establish close relations with the astroglial cells. The repair process leads to the appearance of a newly-formed *glia limitans*, which closely resembles the normal *glia limitans*. This normal component of the surface of the nervous organs is known to be composed of a series of astrocytic prolongations externally lined by a continuous basal membrane, beyond which the connective elements of the leptomeningeal territory are found (84).

Our group has conducted structural studies of the meningeal regeneration process and the peculiar meningo-astroglial relations that are established in the course of the repair of cerebral lesions in rats (121). These experiments show that the appearance of a basal lamina limiting the lesion cavity is a crucial event, for it establishes a clear separation between the mesodermal or neuroectodermal elements. According to our results, patches of electron-dense material similar to the basal lamina are already apparent 10 d after inducing the lesion. After 14 d, practically the entire wound cavity is delimited by a typical basal lamina intimately attached to the underlying nervous parenchyma. This basal lamina always rests upon glial filament-rich cell prolongations identifiable as corresponding to astrocytes. Collagen microfibrils—either isolatedly or forming small bundles—as well as macrophages or regenerative meningocytic elements habitually appear in proximity to the astrocytic prolongations—though always covered by the basal lamina. The *glia limitans* thus regenerated is clearly identifiable 14 d after lesion induction. A feature of this newly-formed *glia limitans* is the marked irregularity of its surface (121); as a result, the meningeal territory frequently presents long solid glial cords, always totally enveloped

by basal lamina, and incompletely covered by thin and delicate meningocytic prolongations (and even somata). Whole astrocyte somata are occasionally seen to form part of the glial cords, and always associated to the corresponding basal lamina cover. This irregularity of the surface of the newly-formed *glia limitans* has been confirmed in different types of lesion (69,122–125). A more recent study by our group (121a) has shown the glial cords to coexpress GFAP and VIM in the meningeal territory.

As regards the appearance of the basal lamina during the lesion repair process, our findings basically coincide with the ultrastructural observations of other authors, though with minor chronological differences (180,127,142,143).

Thus, the glial reaction leading to the appearance of a newly-formed *glia limitans* seems to be similar in response to different types of lesion. As a result of their plasticity and repair capacity, the subpial reactive astrocytes are able to extend their prolongations (and even somata) to the subarachnoid space.

Although ultrastructural observations suggest meningeal elements to induce basal lamina formation by astrocytes and the development of newly-formed *glia limitans*, evidence from experimental models also strongly supports this hypothesis. In astrocyte and meningocyte cultures (144,145), structures similar to *glia limitans* and even irregular deposits of electron-dense material analogous to the basal lamina are seen to form in the contact zones between both types of cell. Experiments have shown that meningocytes may even be essential for *glia limitans* formation: The destruction of meningocytes with 6-hydroxydopamine (6-OHDA) causes disorganization of the *glia limitans* and covering basal lamina (146–149). Meningocyte destruction with 6-OHDA implies a decrease in the concentration of fibrillar collagen types I, III and VI, together with laminin, fibronectin and type IV collagen in relation to the *glia limitans* (135). Moreover, steroids topically applied to cerebral wounds, which would cause a decrease in the proliferation of meningeal elements, has been found to induce a lesser organization of the astrocyte prolongations forming the *glia limitans*, with the appearance of laminin-negative zones at this level (126).

Biochemical studies support the idea that the basal lamina is synthesized by astrocytes. These cells have been shown to produce molecules that form part of the basal lamina, including laminin (127–131), fibronectin (131,132) and proteoglycans (133,134). On the other hand, the meningeal cells are able to produce (135) fibrillar collagen types I, III and VI, fibronectin, laminin, type IV collagen, and heparan-sulfate type proteoglycans. Thus, it seems possible that meningeal cells not only regulate astrocyte synthesis of the basal lamina but also contribute in part to production of the latter. In addition, astrocytes would be able to interact with the extracellular matrix by means of adhesion molecules such as the neural cellular adhesion molecule (NCAM) (136), or tenascin (137–139)—which are likewise expressed by these cells.

6. GROWTH FACTORS AND CYTOKINES

Nonreactive astrocytes possess few growth factors. However, when activated they are known to express multiple factors, cytokines and other substances (140,141). Astrocyte expression of these diffusible compounds varies according to the type of lesion and region involved. Likewise, astrocyte reaction differs between zones close to the lesion and areas located at a distance from the injury site (141).

This section deals with the cytokines and growth factors predominantly implicated in reactive gliosis—fundamentally in traumatic lesions of the CNS.

Three steps may be contemplated in the expression of diffusible factors in post-lesion gliosis, with reference to astrocytes: (a) The origin of the factors that activate astrocytes; (b) The factors expressed by astrocytes in response to injury; and (c) Actions of the diffusible factors secreted by reactive astrocytes upon neighboring structures.

- (a) Astrocyte reaction to injury is a response to different soluble activating factors that in turn induce astrocytes to produce growth factors and cytokines. The source of these triggering substances is the nervous tissue affected by the lesion. Injury itself causes the destruction of many cells, including neurons which release factors that are normally found dissolved in the cytoplasm but are not habitually secreted. An example of such behavior is afforded by the basic fibroblast growth factor (bFGF) (150) and the ciliary neurotrophic factor (CNTF) (151). These two factors, and particularly bFGF, seem to play a special role in astrocyte activation. In addition to destruction of nervous tissue, injury causes vascular rupture, with the subsequent loss of blood-brain-barrier integrity. Additional diffusible factors and cytokines from the extravasated blood plasma (thrombin, platelet-derived growth factor [PDGF], epidermal growth factor [EGF] and insulin, and so on) are thus added and contribute to activate the astrocytes. The extravasated mononuclear cells, together with the microglia, participate in the inflammatory reaction secondary to injury not only by removing the altered tissue debris but also by releasing cytokines such as interleukins (IL) IL-1 and IL-6, interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α) and growth factors such as transforming growth factor-beta (TGF- β) (152–156) and bFGF (157). All these agents induce the expression of specific reactive astrocyte receptors such as bFGFr (158), CNTFr (151), and EGFr (159). The binding of these factors to their corresponding receptors located on the astrocyte membrane, induces the synthesis of further diffusible factors, and thus stimulates and modulates astrocyte response.
- (b) The first sign of astrocyte activation corresponds to the biochemical or *in situ* hybridization detection within the cytoplasm of the mRNA encoding for the different soluble factors. However, astrocyte response does not become effective until the corresponding proteins are synthesized and secreted to the exterior, where they in turn act upon the specific target cells. Once the astrocytes have been activated, a factor synthesis cascade is triggered in sequence over time. In this sense, Cook et al. (157) inform of the existence of both early genes (i.e., which express their mRNA in the first 24 h following injury) and late genes (those with mRNA expression after 72 h).

The early activation of certain genes encoding for soluble factors points to the existence of a rapid astrocyte response, though the first soluble factors to be demonstrated by immunohistochemical techniques appear around three days after the lesion is induced. These factors are bFGF (102), CNTF (151), vascular endothelial growth factor (VEGF) (104), and TGF- β (160–162). Their expression is maintained for prolonged periods of time. Posteriorly, about 5–6 d after injury, the astrocytes express other factors such as insulin-like growth factor-2 (IGF-2) (161), IL-1, IL-6 and TNF- β (154), and nerve growth factor (NGF) (163,164). This latter factor has been detected by Goss et al. (165) after 24 h.

- (c) The factors synthesized by the reactive astrocytes are secreted to the exterior, and act in paracrine fashion upon the neighboring cells (neurons, oligodendroglia or its precursors, endothelium, meningeal cells in the event that destruction affects the surface of the nervous organ, and macrophages located within the lesion site). They also interact with the secreting astrocytes themselves, in an autocrine manner. The action of the diffusible factors always takes place from the exterior; to this effect, it is essential for the factors to be secreted to the exterior, where they in turn bind to specific receptors located on the target cell surface membrane. The fact that astrocytes secrete numerous factors suggests that they play a special role in controlling the nerve tissue repair process, though response

varies according to the region where the lesion is produced. The factors secreted by activated astrocytes act upon many different cells. In this sense, astrocytes may be regarded as the neuralgic center of the repair process, for their activation triggers “explosive” action upon many structures that intervene in the nerve tissue repair process.

These factors exert a protective effect upon the neurons. In this sense, bFGF (157,166), NGF (167), IGF-1 (168) and the leukemia inhibitor factor (LIF) (169) diminish neuronal death in the zones close to the lesion site. However, reactive astrocytes stimulated by IL-1 may exert a neurotoxic effect by producing nitric oxide (170).

Astrocytes are important target cells of factors secreted by their own activated astrocytic counterparts. These factors exert the following effects:

Astrocyte proliferation is stimulated by bFGF (171,172), EGF (173,174) and PDGF (67). Fibronectin, expressed by reactive astrocytes, is a potent stimulator of astrocyte division (175,176). Many factors also inhibit astrocyte proliferation, including IFN- γ (177–179), and TGF- α 1, which inhibits the mitotic effects of FGF and EGF (180). This stimulating and inhibiting behavior of factors produced by the astrocytes themselves supports the idea of their regulatory influence upon gliosis.

Astrocyte hypertrophy, with increases in GFAP and VIM synthesis. These actions are performed by bFGF (171,181–183). However, Reilly et al. (184) observed that bFGF produces a decrease in GFAP mRNA in astrocytes in vitro, whereas TGF- α 1 increases it. Logan et al. (155) described a TGF- α 1 stimulating effect upon GFAP synthesis. CNTF is also a potent inducer of astrocyte hypertrophy (67), stimulating the synthesis of GFAP and VIM—actions that are in turn enhanced by TNF- γ (64,101). According to Kahn et al. (185), CNTF appears to specifically increase astrocyte GFAP synthesis, though without affecting VIM levels.

According to Ridet et al. (141), these soluble factors induce astrocyte synthesis of surface molecules and extracellular matrix. Astrocyte expression of intercellular adhesion molecules (ICAM-1) is stimulated by different growth factors and cytokines such as IL-1 (186). NGF (163) stimulates the synthesis of neural cellular adhesion molecules (NCAM), although interleukin-beta 1 (186) and IFN- γ (140) stimulate ICAM production. Furthermore, NCAM and components of the extracellular matrix such as fibronectin, tenascin or heparan sulfate favor axon growth of the neurons damaged by the wound. However, when astrocytes synthesize other proteoglycans such as keratan sulfate or chondroitin sulfate, such action is effectively inhibited (187,188). Other proteoglycan actions have been reported, including the ability of heparan sulfate to bind to FGF type 4 receptors and stimulate the cell in the absence of growth factor.

Activated astrocyte factors induce astrocyte production of other growth factors and of an increased number of receptors for their own secretory products thereby incrementing reactive astrocyte response to certain trophic factors and cytokines. bFGF induces NGF expression (150,157) and increases the number of bFGF receptors present both in astrocytes and in other cells (157,158,172). Although EGF is not synthesized by reactive astrocytes, the latter do exhibit receptors for this factor (159); as a result, these cells become susceptible to EGF action. In vitro studies have shown EGF to stimulate astrocyte production of bFGF, TGF- β 1 and NGF (140). In turn, TGF- β 1 also induces NGF synthesis in astrocytes (163,189). Interleukin-1 is synthesized by reactive astrocytes (154), and it has been shown to stimulate astrocyte synthesis of NGF (190,191). IGF-1 also stimulates the production of its own receptors on hypertrophic astrocytes (102).

Many of the growth factors synthesized by reactive astrocytes induce these same cells to produce NGF—one of the main functions of which is the induction of a trophic effect upon the damaged neurons, thereby contributing to their survival. In addition, NGF stimulates the synthesis of its own receptors by the same reactive astrocytes (192).

Not all growth factors and cytokines exert a stimulating effect upon astrocytes. Certain diffusible substances synthesized by inflammatory cells and by the reactive astrocytes themselves tend to buffer glial reaction. In this sense, IFN- γ inhibits astrocyte proliferation and the synthesis of extracellular matrix molecules (191). In turn, interleukin-10 (produced by microglia/macrophages) buffers astrocyte reaction (193), with a decrease in bFGF mRNA levels within the astrocytes (194).

Certain growth factors secreted by reactive astrocytes stimulate the proliferation of oligodendroglia precursors in adults (102,157,162). In contrast, Amur-Umarjee et al. (195) have observed an inhibitory effect of astrocytes upon oligodendrocyte remyelination capacity in vitro.

Other growth factors in turn induce vascular proliferation and restoration of the blood-brain-barrier. In this context, bFGF appears to intervene in vessel neoformation (182). VEGF (synthesized by astrocytes and inflammatory cells) seems to stimulate vascular formation following injury (196), while TGF- α 1 would favor reconstitution of the blood-brain barrier (155).

When the lesion affects the surface of the nervous organ, these factors induce neoformation of the *glia limitans*. Logan et al. (155) detected TGF- α 1 in astrocytes, and TGF- α 1 mRNA in meningeal cells.

Astrocytes release factors that stimulate microglial proliferation; these cells concentrate in the lesion site, and in turn increment astrocyte reactivity. This is the case of TGF- α 1 (197) and bFGF (198). In this way a mutual stimulation circuit is established based on the positive feedback principle, between astrocytes and microglia/blood mononuclear cells, thus favoring and reinforcing astrocyte activity in gliosis.

Other elements have also been implicated in the regulation of glial response, including norepinephrine via beta-adrenergic receptor action (91,199), and corticoids (192).

To summarize, the reactive astrocyte plays a prominent role in the regulation and modulation of the scar tissue resulting from damage of the nervous system. This action is largely, though not entirely, mediated by the cascade activation of different growth factors and cytokines, which in turn interact with each other upon the surrounding cells and structures.

7. GLIAL REACTION IN RELATION TO AGE

Most authors consider that astrocyte reaction to lesions of the CNS is less important during the fetal and neonatal period than in adults. In general, immature astrocyte response to injury is similar though less intense than mature astrocyte reaction in the adult (70,152,193,200–202). An important difference in astrocyte response to lesion between the fetal and adult stages is the absence of glial scar formation following CNS injury in the neonatal period (18,21,203,204). However, according to some authors, the response is more dependent upon the type and/or location of the lesion than on astrocyte immaturity. In this sense, glial scars have been described following neonatal injury (70,152,193,200–203).

According to most researchers, the absence of glial reaction in neonatal lesions is a consequence of astrocyte immaturity (205,206). However, Smith et al. (207,208), are of

the opinion that the absence of a glial scar is due to some specific property of the immature astrocytes, and is not a consequence of any cellular incapacity. According to Berry et al. (18a), the absence of a glial reaction could be attributed to the microglia/macrophages rather than to the astrocytes as such. In neonatal animal models, debris elimination is faster and more effective thereby avoiding persistent macrophage presence within the lesion site (152,193,202,206).

A critical period seems to exist in the early neonatal stages, after which the capacity to develop an “adult”-like glial response to CNS injury is acquired. A number of authors have pointed out the coincidence in time between the critical period in which the “adult” response to lesions is acquired and the myelination phase (168,207,208). This coincidence has led to the idea that the presence of myelin and/or oligodendrocyte degradation products determines the formation of a glial scar. However, as has been demonstrated by Berry et al. (18a), lesions are followed by normal healing in myelin-deficient mutants.

The existence of changes in the astrocyte population (particularly hypertrophy, with increased GFAP expression) associated to advanced age is generally accepted. However, unlike in the neonatal period, very few studies have been carried out in individuals of advanced age, and the results obtained are moreover contradictory. According to some authors, astrocyte response in old animals is more intense, extensive, and prolonged in time (209), and proliferation is both greater and earlier (26). In contrast, Kane et al. (46) have observed no increase in astrocyte hypertrophic response in such individuals. In this sense, the conflicting results obtained may be partly attributed to the experimental methodology employed.

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II

NEURON–GLIA INTERCOMMUNICATION

Astrocytes *In Situ* Exhibit Functional Neurotransmitter Receptors

Marilee K. Shelton and Ken D. McCarthy

1. INTRODUCTION

Astrocytes constitute a major portion of brain cells and envelop most neuronal elements. The intimate association of astrocytes and neurons led early anatomists to speculate that astrocytes interact with neurons (1–4). Work beginning in the 1970s determined that astroglia (astrocytes in culture) exhibit a wide variety of neurotransmitter receptors that regulate both second messenger systems and ion channels. These findings suggested that astrocytes *in vivo* might have neurotransmitter receptors enabling them to respond to neuronal activity. The importance, however, of studying astrocytes without culturing them was underscored by reports indicating that the neuroligand responsiveness of astroglia changes in culture (5–7). That placing astroglia in culture altered their phenotype was not surprising given that these cells are generally isolated from their normal cellular and chemical milieu at an early developmental stage and placed into an artificial environment that almost certainly lacks critical developmental cues. Unfortunately, the complex morphology of astrocytes together with their inability to propagate action potentials makes it difficult to study these cells *in vivo* with methods that were so powerful in elucidating the neuronal signaling systems *in vivo*.

Over the past decade, a number of laboratories have carried out studies to determine if astrocytes *in situ* exhibit the plethora of neuroligand receptors expressed by astroglia in culture. Although the results from *in situ* experiments are very limited relative to *in vitro* studies, it has become clear that astrocytes *in situ* exhibit functional neuroligand receptors. Approaches used to study the expression of astrocytic signaling systems *in situ* include immunological staining with receptor-specific antibodies (8), mRNA detection (9), electrophysiological recording of neuroligand responses (10), and analysis of neuroligand-mediated changes in astrocytic Ca²⁺ using Ca²⁺-indicator dyes (11). Each of these approaches has advantages and disadvantages and they frequently complement one another. Here we have attempted to summarize the evidence for the expression of “functional” astrocytic receptors *in situ*; by functional, we are referring to receptors shown to regulate either ion channels or second messenger systems. One caveat in the study of functional astrocytic receptors *in situ* is that astrocytic responses are generally measured in astrocyte perikarya. *In situ* analysis, therefore, may not

detect signaling in astrocyte processes where morphological evidence suggests neuronal-astrocytic interactions may occur. Other caveats in the analysis of functional astrocyte receptors *in situ* are that, generally, few cells are analyzed; and negative findings should be given less value since technical issues with brain slices might prevent detection of functional receptors. In the following sections, we have attempted to summarize the results of studies that focused on the expression of functional astrocyte receptors *in situ*.

2. EVIDENCE FOR FUNCTIONAL GLUTAMATERGIC RECEPTORS ON ASTROCYTES *IN SITU*

Glutamate is the major excitatory neurotransmitter in the brain. It stimulates the ionotropic glutamate receptors (iGluRs), including NMDA and AMPA/kainate receptor subtypes, that gate ion channels. Glutamate also stimulates the metabotropic glutamate receptors (mGluRs) that are coupled to second messenger systems through G proteins. Substantial evidence suggests that astrocytes *in situ* exhibit functional iGluRs and mGluRs.

The first evidence for functional mGluRs on astrocytes *in situ* came from the work of Porter and McCarthy (12). Astrocytes were found to respond to mGluR agonist 1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (t-ACPD) with increases in intracellular Ca^{2+} when studied in the CA1 stratum radiatum region of hippocampal slices prepared from 9–13-day-old (P9-P13) rats (12). Furthermore, responses to t-ACPD were blocked by the mGluR antagonist α -methyl-4-carboxyphenylglycine (MCPG). Studies from other laboratories indicate that treatment with t-ACPD elicits Ca^{2+} increases in astrocytes acutely isolated from the cerebral cortex (7,13) and from astrocytes *in situ* in the visual cortex (14).

An early report indicated that astrocytes in P21–P42 hippocampal slices did not exhibit mGluRs (15). This finding suggested that hippocampal astrocytic mGluRs may serve a developmental role but not be important in the mature hippocampus. We have since demonstrated, however, that mature astrocytes (P30–P35) within hippocampal brain slices have mGluRs coupled to Ca^{2+} increases (16; Fig. 1). tACPD also increased Ca^{2+} levels in GFAP/S100⁺ astrocytes within a ~P66–P71 hippocampal slice (Fig. 2), suggesting that mGluRs exist well into adulthood. Consistent with the detection of functional astrocytic mGluRs *in situ*, immunocytochemical staining suggests that astrocytes within this region express mGluR5, an mGluR subunit coupled to Ca^{2+} increases (17–20). Antibodies for mGluR5 lightly stained astrocytes in adult hippocampus and cortex (19). Interestingly, mGluR5-immunoreactive astrocytes in the hypothalamus envelop presynaptic boutons, where they might bind neuronally-released glutamate (17).

The CA1 stratum radiatum region of the hippocampus receives excitatory glutamatergic input from the pyramidal neurons of CA3 via Schaffer collateral processes. The Schaffer collateral-CA1 pyramidal neuron (SC-CA1) synapse is one of the most studied synapses in brain. Experiments carried out by Porter and McCarthy (21) indicate that stimulation of this pathway leads to astrocytic Ca^{2+} responses that are blocked by

1. tetrodotoxin (which blocks action potential propagation),
2. conotoxin MVIIC (which blocks neurotransmitter release in this region) and
3. the mGluR antagonist, MCPG.

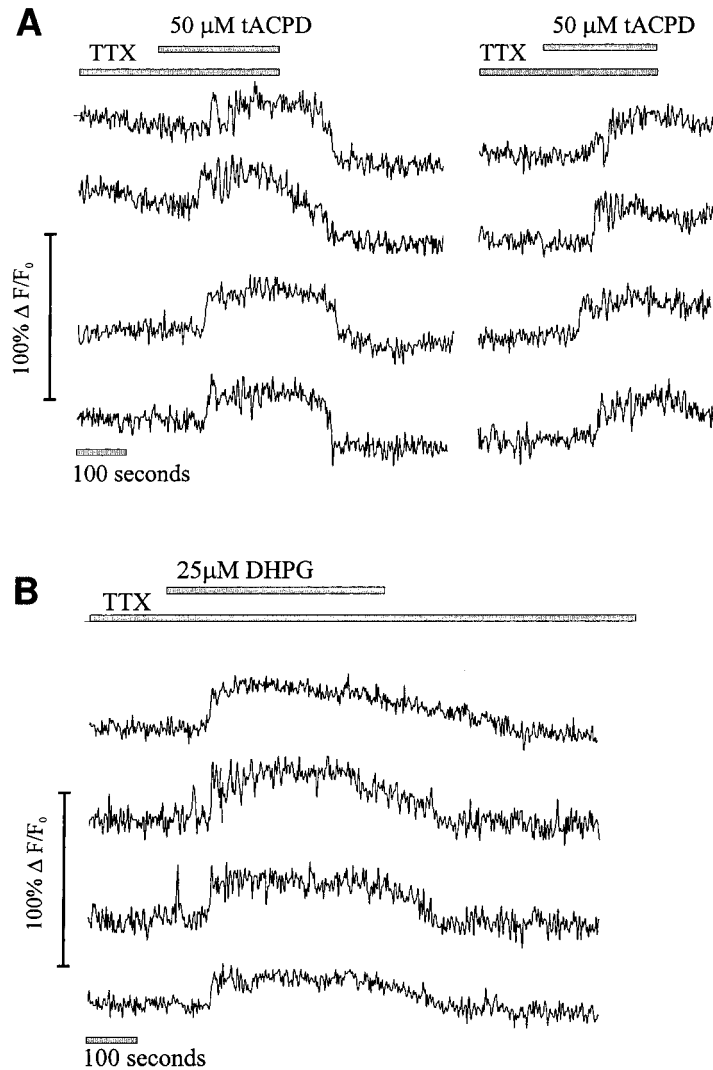


Fig. 1. Mature astrocytes in the CA1 stratum radiatum region of acutely-isolated hippocampal brain slices respond to metabotropic glutamate agonists with calcium increases. Fluorescence increases represent increases in intracellular calcium, as measured after loading cells with the Ca^{2+} sensitive dye Calcium Green-1 AM. Traces shown are from cells that were immunologically identified as astrocytes by immunocytochemically staining slices for astrocyte markers GFAP and S100. Traces are arbitrarily separated along the y-axis. Slices were pretreated with 1 μM tetrodotoxin (TTX) to prevent stimulation of action potentials. **(A)** P31 astrocytes exhibit calcium increases in response to metabotropic glutamate receptor agonist 1-aminocyclopentane-trans-1,3-dicarboxylic acid (t-ACPD). **(B)** P32 astrocytes exhibit calcium increases in response to the metabotropic glutamate receptor agonist dihydrophenylglycine (DHPG). Shelton, M.K. and McCarthy, K.D. (1999) Mature hippocampal astrocytes exhibit functional metabotropic and ionotropic glutamate receptors in situ. From Shelton and McCarthy 1999, (16) with permission.

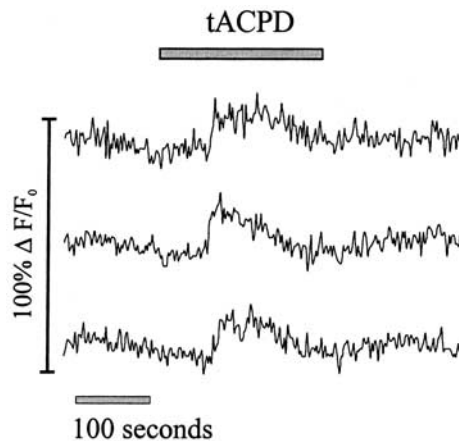


Fig. 2. Mature astrocytes, in the CA1 stratum radiatum region of ~P66–P71 acutely-isolated hippocampal brain slices, respond to the metabotropic glutamate receptor agonist tACPD with calcium increases. Shelton and McCarthy, unpublished results.

Other investigators have subsequently verified and extended these observations by suggesting that increasing Ca^{2+} in these astrocytes triggers them to release glutamate that subsequently increases Ca^{2+} in CA1 pyramidal neurons (14). Overall, it is clear from these studies that hippocampal astrocytes express functional mGluRs that respond to applied mGluR agonists as well as glutamate released by synaptic activation.

There is also convincing evidence that astrocytes exhibit functional iGluRs. The AMPA/Kainate iGluRs (AMPA/KA-Rs) consist of the AMPA-preferring (AMPA-Rs) and kainate-preferring receptors (KA-Rs). AMPA-Rs and KA-Rs are collectively referred to as AMPA/KA-Rs, or non-NMDA receptors, since many reports do not distinguish between these two receptor types. Reports demonstrate that AMPA/KA-Rs stimulate depolarization and/or Ca^{2+} elevations in freshly-isolated and *in situ* astrocytes, as well as presumed astrocyte progenitors (10,12,22–27). As with mGluRs, an initial report suggested that mature (P21–P42) hippocampal astrocytes lack AMPA/KA-Rs (15). Subsequent studies from two separate laboratories, however, indicate that mature astrocytes express functional AMPA/KA-Rs coupled to depolarization (28) and Ca^{2+} increases (16, Fig. 3). AMPA/KA-R stimulation affected astrocytes both when they were isolated from slices and when neuronal action potentials were blocked, indicating that AMPA/KA-R agonists were directly affecting astrocytes (10,12,28) (Shelton and McCarthy, Neurochemistry). When AMPA/KA-R responses have been closely examined, they appear to occur via AMPA-Rs in hippocampal cells presumed to be astrocyte progenitors (10,28).

In addition to the physiological evidence for AMPA/KA-Rs, there is also evidence that astrocytes express AMPA-R and KA-R subunits. AMPA-Rs are composed of four subunits named GluR1–4, and KA-Rs are composed of subunits GluR5–7 and KA1–2. GluR4 and GluR1 receptors or mRNA have been localized to astrocytes from cerebellum, hippocampus, cerebral cortex, and several other brain regions (9,30–43). Bergmann glia, a type of specialized astrocyte, do not appear to express GluR2 mRNA (33,44). This is in contrast to the detection of GluR2 transcripts in each presumed astrocyte progenitor examined from the hippocampus (9). The GluR2 expression is likely to control Ca^{2+}

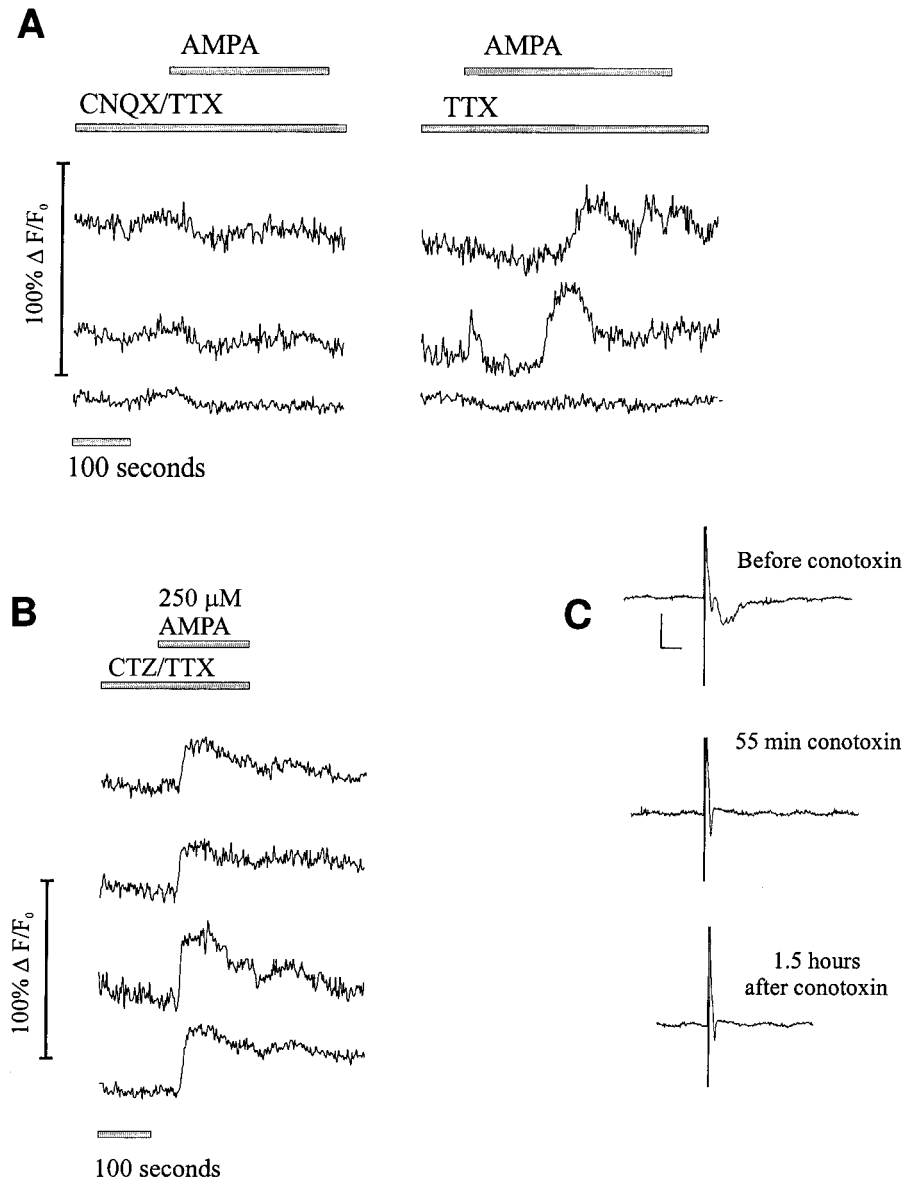


Fig. 3. Mature astrocytes in the CA1 stratum radiatum region of acutely-isolated hippocampal brain slices respond to the non-NMDA receptor agonist AMPA with calcium increases. (*see* Fig. 1) Slices were treated with cyclothiazide (CTZ) to prevent desensitization of α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-preferring non-NMDA receptors. **(A)** Astrocytes do not respond to AMPA in the presence of the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). Following CNQX washout, AMPA elicits Ca^{2+} responses. **(B)** P35 astrocytes respond to AMPA after treatment with 5 μM conotoxin MVIIC to prevent neurotransmitter release. **(C)** Conotoxin blocks postsynaptic field potentials, indicating that neuronal release is inhibited by conotoxin treatment. From (16), with permission.

influx through these receptors, as it does in neurons (45). Although functional KA-Rs have not been detected on astrocytes *in situ*, limited immunocytochemical evidence suggests that astrocytes express these receptors (42,46). Perhaps immunocytochemical staining detects astrocyte KA-R subunits that are difficult to detect physiologically because they rapidly desensitize or are masked by AMPA-R activation.

The third type of iGluRs are the NMDA receptors (NMDA-Rs), which are permeable to Na⁺, K⁺, and Ca²⁺. NMDA-R mediated Ca²⁺ elevations are not usually detected on astrocytes in culture or *in situ* (47–50). There is some evidence, however, that NMDA depolarizes acutely-isolated astrocytes and astrocytes *in situ*. NMDA elicited small, atypical currents in Muller cells (51) and Bergmann glia (47), and also depolarized a subpopulation of hippocampal glial cells (25). There is also limited evidence that astrocytes express NMDA-R subunit transcripts or receptor proteins. For example, Muller cells, Bergmann glia, cortical astrocytes, and thalamic astrocytes may express one or more NMDA-R subunit transcripts or receptors (52–56). Together, the data suggest that subpopulations of astrocytes may express NMDA-Rs but that these may differ from neuronal NMDA-Rs.

Interestingly, stimulation of the Schaffer collateral pathway at levels that consistently activate astrocytic mGluRs, fails to elevate Ca²⁺ via iGluRs (21). In contrast, when 4-aminopyridine (4-AP) is included in the perfusion buffer, stimulation of the Schaffer collateral pathway leads to responses that can only be blocked by the combination of both mGluR and iGluR antagonists. 4-AP is a potassium channel blocker that is known to increase the duration of neurotransmitter release and prolong synaptic transmission. It is possible that astrocytic iGluRs are located farther from sites of glutamate release and require higher levels of stimulation to be activated. This is interesting and may reflect a mechanism whereby astrocytes respond differently to varying levels of neuronal activity.

2.1. GABA Receptors

γ -aminobutyric acid (GABA) is the major inhibitory neurotransmitter in mammalian brain. GABA stimulates GABA_B receptors (GABA_BRs) that are coupled to Ca²⁺ channels, K⁺ channels and adenylyl cyclase through G proteins (56A). GABA also stimulates GABA_A and GABA_C receptors (GABA_ARs, GABA_CRs) that gate Cl⁻ channels. Astrocytes depolarize when Cl⁻ channels are opened because their high intracellular [Cl⁻] creates an outward Cl⁻ gradient. Initial studies demonstrated that GABA depolarizes cultured astroglia (57). GABA_ARs have since been reported to depolarize acutely isolated and *in situ* astrocytes from hippocampus, cerebellum, and pituitary (58–60). GABA not only depolarizes astrocytes, but may also elevate Ca²⁺ if astrocytes are sufficiently depolarized to open voltage-dependent Ca²⁺ channels (VDCC). For example, GABA induced [Ca²⁺]_i elevations that were depressed by VDCC antagonists in hippocampal astrocytes (60). It should be mentioned, however, that data from another laboratory suggests that hippocampal astrocytes do not exhibit VDCC (15). Interestingly, the function of GABA_ARs may be developmentally regulated. This is suggested by observations that GABA_ARs depolarize optic nerve and cerebellar astrocytes less as animals mature (59,61). In addition to functional evidence for GABA_AR-mediated signaling in astrocytes, there is also evidence that astrocytes express several of the GABA_AR α , β , γ , and δ subunits. Hippocampal astrocytes were labeled by antibodies for α_1 and β_1 subunits (60).

Interestingly, α_2 , α_3 , and δ subunits are transiently expressed at about the same age that GABA_ARs mediate large depolarizations in Bergmann glia (59). Subunit expression and GABA-mediated depolarizations both decreased with maturation (59). It may be that GABA_ARs are only transiently expressed because they are important in development.

Importantly, GABA_AR channels may be opened by neuronally-released GABA. Electrically stimulating stalk neurons in isolated pituitaries evoked depolarizations in stellate pituicytes (a specialized type of astrocyte; (58)). Blocking action potential propagation or GABA_A receptors with TTX or bicuculline, respectively, blocked electrically-induced astrocytic depolarizations, suggesting that they depend on neuronal activity and GABA_ARs.

In addition to ionotropic GABA_ARs, evidence also suggests that neuronal activity stimulates astrocytic GABA_ARs. For example, direct stimulation of inhibitory neurons leads to Ca²⁺ elevations in neighboring hippocampal astrocytes (62). These Ca²⁺ elevations were blocked by the potent GABA_BR antagonist CGP55845A (62,63), suggesting that they occur through GABA_BRs. Furthermore, GABA and GABA_BR agonist baclofen elicited astrocyte Ca²⁺ responses that were blocked by CGP55845A but not by the GABA_AR antagonist bicuculline (62). These Ca²⁺ responses to baclofen and GABA depended on extracellular Ca²⁺ suggesting that a Ca²⁺ influx was necessary for GABA_B-mediated increases in astrocytic Ca²⁺. Together, these data suggest that astrocyte GABA_BRs mediate Ca²⁺ elevations that depend on Ca²⁺ influx. A puzzling aspect of these findings is that GABA_B receptors are not typically associated with increased Ca²⁺ influx. Another report, however, suggests that cortical astroglia may also exhibit GABA_BR-mediated Ca²⁺ responses that depend on extracellular Ca²⁺ (64). Perhaps astrocytes have a unique GABA receptor that is mediating these responses.

2.2. Adrenergic Receptors

Norepinephrine and epinephrine are important modulatory neurotransmitters within the central nervous system (CNS) that stimulate α and β adrenergic receptors (α ARs and β ARs). It has long been known that cultured astroglia express both α_1 - and α_2 -adrenergic receptors (65–67). There are several lines of evidence suggesting that astrocytes *in situ* also express α ARs. For example, norepinephrine or the α_1 AR-selective agonist phenylephrine evoked Ca²⁺ elevations in Bergmann glia and hippocampal astrocytes of acutely isolated brain slices (15,50,68). These Ca²⁺ elevations were inhibited by the α_1 AR selective antagonist prazosin, but they were not affected by α_2 AR or β AR ligands (15,68). Astrocyte responses were not secondary to transmitter release from neurons, since neither isolating astrocytes away from intact brain tissue, nor treating slices with tetrodotoxin (TTX) prevented α_1 AR-mediated responses (15,50,68). α_1 Rs were also localized to acutely-isolated astrocytes with a radiolabeled α_1 AR antagonist (69,70).

In addition to α_1 AR, two reports suggest that α_2 -ARs are present on astrocytes *in situ*. Antibodies for α_2 ARs reportedly bound astrocytes in hippocampus (71) and locus ceruleus (72). Interestingly, these immunoreactive astrocytes were observed near dendrites and terminals of catecholamine neurons (71,72), where they might bind released norepinephrine.

A number of ligand binding and immunocytochemical reports also localize β ARs to astrocytes of different brain regions (8,27,70,73–76A). For example, radiolabeled β AR

ligands bound to protoplasmic and fibrous astrocytes that were acutely-isolated from cerebral cortex, striatum, cerebellum, and trigeminal motor nucleus (70,73). Aoki and colleagues combined β AR immunostaining with electron microscopy to assess whether astrocytes express β ARS. They concluded that β ARs are expressed by astrocytes in visual cortex (74,76A) and cerebral cortex (8). They also observed β AR positive astrocytes closely associating with axon terminals, dendrites, and synaptic junctions of catecholamine neurons (74). In summary, astrocytes in various brain regions appear to express α_1 , α_2 and β -adrenergic receptors. These receptors have been observed where they are likely to be exposed to neuronally-released norepinephrine (71,72,74).

2.3. Acetylcholine Receptors

There is also evidence that astrocytes have G protein-coupled muscarinic cholinergic receptors (mAChRs). mAChR stimulation increases Ca^{2+} in immunologically-identified astrocytes in acutely-isolated hippocampal slices (77a). These Ca^{2+} increases were inhibited by the mAChR antagonist pirenzepine, suggesting that the M1 mAChR subtype may be involved. In addition to physiological studies *in situ*, hippocampal and cortical astrocytes are labeled by an antibody that does not distinguish among mAChR subtypes (77–80). Interestingly, this immunoreactivity increased in the hippocampus when seizure-like activity was induced (78), hinting that neuronal activity might alter astrocyte mAChR expression. Finally, the number of astrocytes stained by this M35 antibody markedly increased in aged rats, compared to young rats (80,81). It is not clear if this reflects astrocyte proliferation/gliosis with aging, increases in the number of receptors per astrocyte, or changes in the availability of the epitope recognized by the antibody. The same antibody reportedly labeled gray matter astrocytes intensely in some Alzheimer's diseased brain (82). It is interesting to speculate that intense mAChR immunoreactivity in astrocytes from Alzheimer's brain reflects an attempt on the part of the astrocytes to compensate for cholinergic neuron loss associated with this disease. In summary, it seems quite clear that mammalian astrocytes *in vivo* express mAChRs.

2.4. Histamine Receptors

Histamine is a modulatory neurotransmitter that influences physiological states such as wakefulness and behaviors such as learning and aggression (83). H1 and H2 receptors are associated with cultured astroglia (84,85). *In situ*, histamine elicited Ca^{2+} increases in Bergmann glia, immunologically-identified hippocampal astrocytes, and glial precursor cells in the corpus callosum (68,77a,86). H1 receptors probably mediate hippocampal astrocyte and Bergmann glia Ca^{2+} responses, as shown by their sensitivity to H1-selective antagonist chlorpheniramine (68), Shelton & McCarthy, submitted). Bergmann glia responses were dependent on intracellular Ca^{2+} release and were not mimicked by H2 or H3 agonists. (68). In corpus callosum, histamine elicited Ca^{2+} elevations in P3–P7 cell bodies that were probably glial precursors (86). Histamine, however, rarely elicited responses in P11–P18 corpus callosum cell bodies that are primarily glial precursors and astrocytes (86). In summary, hippocampal astrocytes and Bergmann glia *In situ* express histamine receptors coupled to Ca^{2+} increases.

2.5. Adenosine and P2 Receptors

A variety of studies suggest that ATP and adenosine can act as traditional neurotransmitters that are released from neurons and activate signaling systems through surface receptors (87,88). Adenosine stimulates a family of receptors known as P1 receptors, whereas ATP and related substances stimulate a family of receptors known as P2 receptors. Adenosine stimulates Ca^{2+} elevations in hippocampal astrocytes *in situ* and freshly isolated cortical astrocytes (11,89). Pharmacological sensitivities suggest that Ca^{2+} elevations in hippocampal and cortical astrocytes occur by activation of $\text{A}_{2\text{B}}$ adenosine receptors (11,89). Adenosine activates Ca^{2+} release through intracellular stores, since responses depend on phospholipase C activity but not on extracellular Ca^{2+} (11,89). As responses were observed either with TTX or in isolated astrocytes, it is unlikely that they are secondary to neuronal stimulation (11,89). $\text{A}_{2\text{B}}$ adenosine receptors, however, may not be the only P1 receptor subtype on astrocytes *in situ*. Some hippocampal astrocytes responded to an agonist that can activate $\text{A}_{2\text{A}}$ and A_3 receptors, suggesting one or both of these receptors may also be on astrocytes (11).

In addition to adenosine receptors, astrocytes also have P2 receptors that are activated by ATP and other nucleotides. Both ionotropic and metabotropic P2 receptors have been observed on cultured astroglia (85). Studies suggest that astrocytes *in situ* may also express P2 receptors. For example, ATP elicited Ca^{2+} increases that were inhibited by the P2 antagonist suramin in Bergmann glia. These Ca^{2+} elevations were not accompanied by conductance changes, suggesting that P2Y metabotropic receptors were involved (90). Hippocampal astrocytes also appear to have P2 receptors since the P2Y₁ agonist, ADP β S, elicited Ca^{2+} elevations in about 20% of astrocytes examined (11). ADP β S evoked responses in the presence of TTX, suggesting that ATP directly stimulates astrocyte receptors (11). In contrast to P2Y receptors, ionotropic P2X receptors have not been directly detected on astrocytes *in situ*. For example, ATP did not elicit conductance changes in electrically recorded hippocampal astrocytes (91) or Bergmann glial *in situ* (90). Neal et al., however, suggest that P2X receptors might modulate GABA uptake into retinal glial cells (92). In summary, ATP, adenosine and other nucleotides can signal to astrocytes *in situ* through P2 and A_2 adenosine receptors.

2.6. Serotonin Receptors

Seven classes of serotonin receptors (5HT) have been identified. Cultured astroglia appear to have 5HT₂ receptors coupled to Ca^{2+} (93,94). In contrast to astroglia, there is little evidence for functional 5HT receptors on astrocytes *in situ*. Serotonin did not elicit Ca^{2+} elevations in Bergmann glia *in situ* or in acutely isolated cortical or hippocampal astrocytes (6,7,13,68,95). 5HT receptors were expressed when acutely-isolated astrocytes were cultured in serum containing medium, suggesting that culturing 5HT receptors astroglia may induce 5HT receptor expression (6,7). Serotonin did elicit a small number of responses in corpus callosum glial precursors *in situ*; however, these responses occurred less frequently as the precursors develop into astrocytes and oligodendrocytes (86). Although astrocytes do not appear to express the Ca^{2+} -elevating 5HT₂ receptors until they are cultured, they may express 5HT_{1A} receptors. Azmitia et al. report that immunostaining colocalizes 5HT_{1A} receptors and GFAP in septum, hippocampus, cerebral cortex, and other regions (95,96). In these studies, 5HT immunostaining varied substantially in the different brain regions examined (95). In summary,

there is little evidence to date that astrocytes *in situ* express 5HT receptors coupled to Ca^{2+} increases; however, they may express other types of 5HT receptors.

2.7. Opioid Receptors

Opioid receptors δ , κ , and μ are activated by certain endogenous peptides such as β -endorphin, enkephalins, endomorphins, and/or dynorphin A. In general, opioid receptors modulate adenylate cyclase activity, increase K^+ channel activity, and inhibit Ca^{2+} channel activity through G proteins (97). There is limited evidence that opioid receptors are on astrocytes *in situ*. For example, the δ receptor agonist [D-Pen5, D-Pen5]-enkephalin elevated Ca^{2+} in several acutely-isolated cells presumed to be astrocytes, and δ receptor antagonist ICI 174.864 blocked these Ca^{2+} elevations (13). As the Ca^{2+} source was not investigated, the receptor signaling mechanism responsible for Ca^{2+} increases in these studies remains uncertain (13). Ligand binding studies suggest pituicytes *in situ* may express κ opioid receptors. In these studies, neuronal innervation was removed from the pituitary by transecting pituitary stalks. Opioid binding sites that remained after neuronal degeneration were attributed to the expression of these receptors by pituicytes (98,99). In summary, it seems that certain populations of astrocytes express opioid receptors *in situ*. Additional studies, however, are required to verify that nonspecialized astrocytes express opioid receptors.

3. ROLE OF ASTROCYTE NEUROTRANSMITTER RECEPTORS

The results of *in vitro* studies carried out over the past three decades show that astrocytes have the potential to express most neurotransmitter receptors. Unfortunately, it has been far more difficult to establish the repertoire of neurotransmitter receptors expressed by astrocytes *in situ*. To date, only a small percentage of the receptors shown to be expressed by astroglia in culture are known to be expressed by astrocytes *in situ*. Furthermore, we know little about the spatial distribution of astrocytic receptors or the effects of development and aging on the expression and distribution of astrocytic signaling systems. Less is known about astrocytes *in situ* primarily because it is more difficult to study astrocyte signaling systems *in situ*. It is clear, however, that astrocytes *in situ* express functional receptors that are linked to second messenger systems and ion channels. Importantly, in certain situations, it has been possible to demonstrate that astrocytic receptors are activated during neuronal activity. The emerging story suggests that astrocytes are likely to express a similar array of neurotransmitter receptors as neurons. The expression and activation of these receptors may vary both spatially and temporally throughout brain. It is possible that functional microdomains of astrocytic syncytia interact with local neuronal elements without involving perikarya where astrocyte responses *in situ* are monitored. If this occurs, it is conceivable that individual astrocytes exhibit marked receptor heterogeneity among their microdomains and that the complement of receptors expressed in each domain depends on the types of neurotransmitters released in that specific location. Developing new methods to study astrocytic signaling within astrocytic microdomains may be critical for us to understand the significance of bidirectional communication between neurons and astrocytes in the developing, mature, and aging brain.

The prevailing view is that the activation of astrocytic signaling systems may support and modulate neuronal signaling and function. A few of the different ways that astrocytic signaling systems may influence neurotransmission are discussed below.

Although we are a very long way from understanding the role of astrocytes in brain function, there is a growing consensus among neurobiologists that astrocytes are likely to play a much greater role in neurophysiology than imagined only a few years ago.

Astrocytes *in situ* are known to express receptors linked to most of the known second messenger systems including cyclic AMP, cyclic GMP, Ca^{2+} , and NO. Furthermore, astrocytes exhibit a number of receptors that directly or indirectly regulate the opening and closing of ion channels. Collectively, these different signaling systems affect a wide variety of processes that could markedly influence the composition of the extracellular space and in turn, neurotransmission. Receptor stimulation, for example, changes astroglial volume *in vitro*. *In vivo*, such an astrocyte volume change could alter the extracellular volume and thereby influence local ion and neurotransmitter concentrations, as well as the distances between neurotransmitter release sites and target receptors. Since modulating K^+ , Ca^{2+} , or neuroligand concentrations affects neuronal activity, these volume changes could affect neurotransmission. Astrocytes might also modulate $[\text{K}^+]_o$ by changing their capacity to buffer $[\text{K}^+]_o$. Indeed, the conductance of the astrocytic K^+ channels and gap junctions thought to be involved in K^+ buffering can be regulated *in vitro* through signaling pathways stimulated by neuroligands (100,101). If astrocyte microdomains respond to neurotransmitters independently, they could selectively alter the neuronal environment at particular spines.

In addition to influencing the neuronal environment by changing their volume and K^+ buffering, astrocyte receptor stimulation may also affect the uptake of neuroligands released during synaptic activity. It has become clear over the past several years that astrocytes also exhibit transport systems that are important in removing neurotransmitters (102–105). For example, astrocytes express glutamate transporters that may participate in terminating the effects of synaptically released glutamate. Barbour et al. and Yu et al. have reported that the uptake of glutamate into astrocytes is affected by arachidonic acid (106,107), a product generated following the activation of certain astrocytic glutamate receptors (108). It is easy to imagine that regulating the removal of glutamate by astrocytes could markedly affect neuronal activity in a spatially restricted manner.

Astrocytes could also affect neuronal activity by slowly changing neuronal morphology and connections. Studies suggest that neuroligands stimulate astrocytes *in vitro* to synthesize neurotrophic factors (109,110). Perhaps neuronal plasticity involves the activation of astrocytic receptors that regulate the synthesis and release of neurotrophic factors. The release of these neurotrophic factors could induce dendritic remodeling and long term changes in neuronal connections. Again, this could occur in a spatially-restricted manner.

In addition to ways that astrocytes might indirectly modulate neuronal communication, recent findings suggests that astrocytes could affect neuronal activity rather directly. Several laboratories have presented data suggesting that astrocytes release glutamate in a Ca^{2+} -dependent manner. Ca^{2+} -triggered neurotransmitter release from astrocytes was first demonstrated in cocultures of neurons and astroglia. In these experiments, astroglia released glutamate in response to several stimuli that elevated $[\text{Ca}^{2+}]_i$. This glutamate release stimulated Ca^{2+} in neighboring neurons and affected their activity through neuronal GluRs (111–113).

Experiments also suggest that astrocytes *in situ* release glutamate in response to neuroligands that increase $[\text{Ca}^{2+}]_i$. To date, astrocyte Ca^{2+} responses in hippocampus, visual

cortex and retina have been reported to affect neurons through what appears to be a glutamate release. In one study, the mGluR agonist tACPD elicited neuronal Ca^{2+} elevations that were dependent on the activation of iGluRs (14). Through a series of experiments, the investigators made a convincing case that the neuronal iGluR-mediated Ca^{2+} responses were due to glutamate release from surrounding astrocytes (14). Kang et al. also reported that increasing astrocyte Ca^{2+} influenced pyramidal neurons (62). In this study, electrically stimulating astrocytes increased the frequency of miniature inhibitory postsynaptic currents (mIPSCs) in pyramidal neurons. Additional experiments were consistent with the conclusion that Ca^{2+} elevations in astrocytes triggered them to release glutamate that stimulates iGluRs on GABAergic neurons and that GABAergic neurons then increase their release of GABA onto pyramidal neurons (62). Retinal astrocytes also appear to modulate synaptic activity *in situ* (114). In these experiments, glial Ca^{2+} waves often decreased evoked neuronal spiking. As above, it appeared that increasing astrocytic Ca^{2+} led to the release of glutamate which then stimulated inhibitory neurons and decreased the evoked spiking of other neurons (114). Collectively, the findings suggest that astrocytes release glutamate that modulates neuronal activity. Furthermore, it appears that astrocytic glutamate release can enhance or inhibit synaptic transmission, depending on which neurons are stimulated. These findings suggest that astrocytes may modulate synaptic transmission *in situ*. The impact of the release of neuroactive molecules by astrocytes on neurotransmission will be a very exciting area of investigation for some time to come.

Very little is known about the role of astrocyte receptor signaling during development and aging. It seems likely that expression and astrocyte receptor signaling does change throughout life. It is easy to imagine a role for astrocytic receptor signaling during development, where receptor stimulation could affect the secretion of neurotrophic factors or expression of adhesion molecules important in neuronal migration and differentiation. In the adult brain, it may be that astrocytic receptor signaling systems serve primarily to subtly modulate neurotransmission. It seems premature to speculate about how the process of aging affects astrocytic receptor signaling until we know more about the expression and role of astrocytic receptors in adult brain. However, given that the morphology and number of astrocytes change during aging, it seems likely that the impact of astrocytic receptor signaling systems will also change.

4. CONCLUSION

To conclude, it appears that astrocytes express a variety of neuroligand receptors whose activation directly affects neuronal excitability. The challenge will be to develop new methods for determining what conditions allow astrocytic receptor activation and for determining the functional outcome of activating astrocytic receptors. It seems likely that both high resolution imaging of astrocytic-neuronal interactions at the synapse and molecular disruption of specific astrocytic signaling molecules will be required to sort out the role of astrocytes in neurophysiology and brain function.

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Glia and Extracellular Space Diffusion Parameters in the Injured and Aging Brain

Eva Syková

1. INTRODUCTION

The extracellular space (ECS) is the microenvironment of the nerve cells and an important communication channel (1–5). It includes ions, transmitters, metabolites, peptides, neurohormones, other neuroactive substances and molecules of the extracellular matrix, and directly or indirectly affects neuronal and glial cell functions. Populations of neurons can interact both by synapses and by the diffusion of ions and neurotransmitters in the ECS. Since glial cells do not have synapses, their communication with neurons is mediated by the diffusion of ions and neuroactive substances in the ECS.

Neurons and glia release ions, transmitters, and various other neuroactive substances into the ECS. Substances which are released nonsynaptically diffuse through the ECS and bind to extrasynaptic, usually high-affinity, binding sites located on neurons, axons, and glial cells. This type of extrasynaptic transmission is also called “diffusion transmission” (neuroactive substances diffuse through the ECS) or “volume transmission” (neuroactive substances move through the volume of the ECS) (4–7). Neuroactive substances can diffuse through the ECS to target neurons, glial cells or capillaries. This mode of communication without synapses can function between neurons (even those far distant from release sites), as well as between neurons and glial cells, and may provide a mechanism of long-range information processing in functions such as vigilance, sleep, chronic pain, hunger, depression or plastic changes and memory formation. The size and irregular geometry of diffusion channels in the ECS (tissue tortuosity and anisotropy, *see Subheading 2.*) substantially affect and/or direct the movement of various neuroactive substances in the CNS (Fig. 1) and thereby modulate neuronal signaling and neuron-glia communication. Changes in the ECS diffusion parameters may, therefore, result in impairment of the signal transmission and contribute to functional deficits and to neuronal damage. Dynamic changes in ECS ionic composition, volume, and geometry accompany neuronal activity, glial development and proliferation, aging, and some brain pathological states.

2. ECS Composition

Cellular elements and blood vessels fill about 80% of the total CNS tissue volume and the remaining portion (15–25 %) is the ECS. ECS ionic changes resulting from

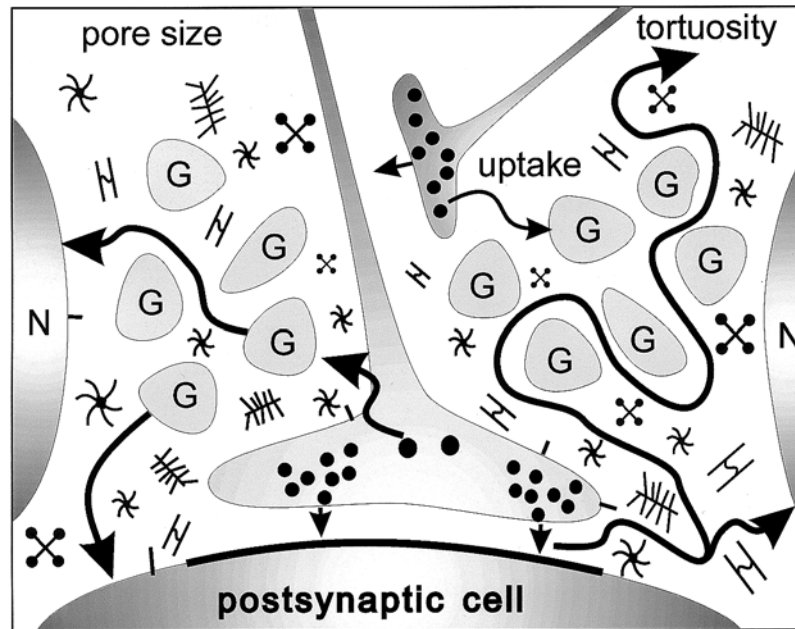


Fig. 1. Schematic of CNS architecture. The CNS architecture is composed of neurons (N), axons, glial cells (G), cellular processes, molecules of the extracellular matrix and intercellular channels between the cells. The architecture affects the movement (diffusion) of substances in the brain, which is critically dependent on channel size, extracellular space tortuosity and cellular uptake.

transmembrane ionic shifts during neuronal activity depolarize neighboring neurons and glial cells, enhance or depress their excitability, and affect ion channel permeability (1,3,4,8,9,10). These ionic changes may also lead to the synchronization of neuronal activity and stimulate glial cell function.

In the mammalian CNS, the average ionic constituents of the ECS are basically the same as in the cerebrospinal fluid: About 141 mM Na⁺, 124 mM Cl⁻, 3 mM K⁺, 121 mM HCO₃⁻, 1.2 mM Ca²⁺ and about 2.5 mM Mg²⁺. However, *in vivo* measurements with ion-selective microelectrodes have revealed local changes in ECS ionic composition resulting from neuronal activity. The local changes in ion activity are localized to areas of high spontaneous activity (Fig. 2) (11,12), or in areas being activated by adequate stimuli, e.g., tactile, visual, auditory, taste, aversive and painful stimuli (13–17).

An activity-related increase in extracellular K⁺ activity ([K⁺]_e), alkaline and acid shifts in extracellular pH (pH_e), and a decrease in extracellular Ca²⁺ concentration ([Ca²⁺]_e) have been found to accompany neuronal activity in a variety of animals and brain regions *in vivo*, as well as *in vitro* (1,3,4,9). After sustained adequate stimulation or after repetitive electrical stimulation of the afferent input, the ionic changes reach a certain steady state, the so-called “ceiling” level (Fig. 2). The K⁺ ceiling level in mammalian brain or spinal cord is 7–8 mM K⁺; the alkaline shifts do not exceed 0.02 pH units and the acid shifts 0.25 pH units. There is now convincing evidence for the neuronal origin of the extracellular alkaline shift and the glial origin of the activity-related acid shift. After tetanic stimulation of the sciatic nerve (30–100 Hz), the [K⁺]_e ceiling

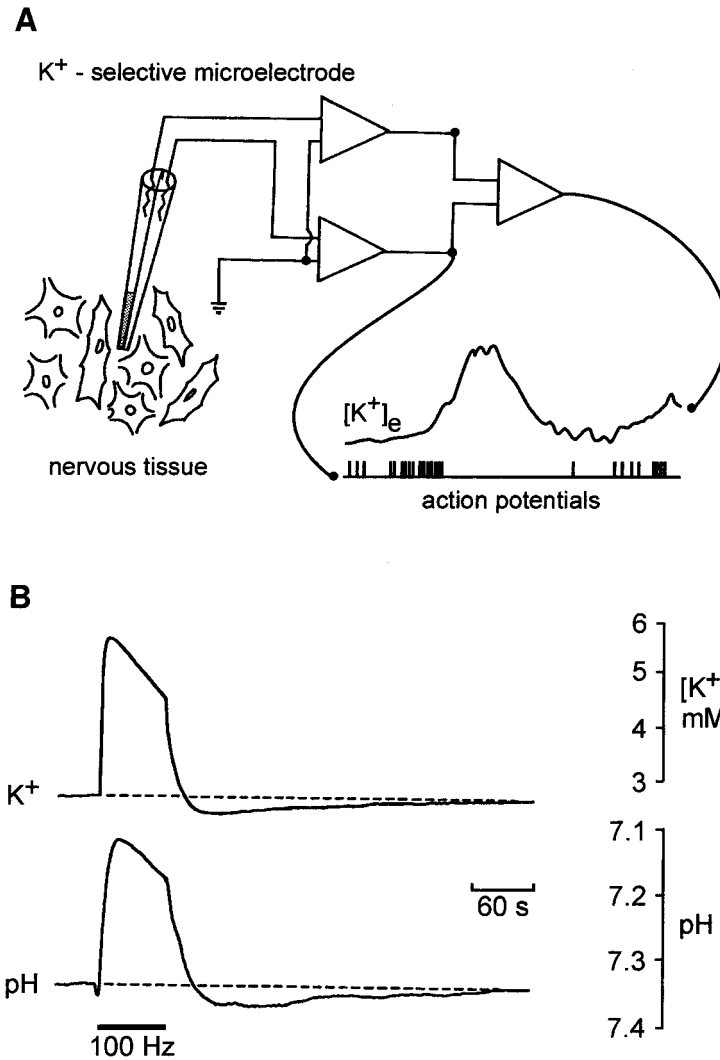


Fig. 2. (A) Diagram of experimental set-up for simultaneous recording of $[K^+]_e$ and action potentials with a double-barrelled K^+ selective microelectrode. Elevation of $[K^+]_e$ in the unstimulated reticular formation of a rat is associated with spontaneous bursts of cell firing recorded by the reference barrel. (B) Stimulation-evoked pH_e and $[K^+]_e$ changes in the spinal dorsal horn of a rat. The stimulation of the dorsal root at a frequency of 100 Hz evoked an increase in $[K^+]_e$. The change in pH_e was biphasic; first a small and fast "initial" alkaline shift occurred, which was followed by a dominating acid shift. Note the poststimulation undershoots in $[K^+]_e$ as well as in pH_e .

level in the adult rat is attained in 5–8 s, whereas the ceiling level of the acid shift is reached in 10–20 s. When stimulation is continued beyond this, a gradual decrease of both transients, $[K^+]_e$ and pH_e , occur after the ceiling levels are reached because of homeostatic mechanisms in neurons and glia (Fig. 2). Extra- and intracellular K^+ and pH homeostasis is ensured by a number of mechanisms in adult brain (1,3,8,9,10). However, homeostasis is not so efficiently controlled during development, when glial

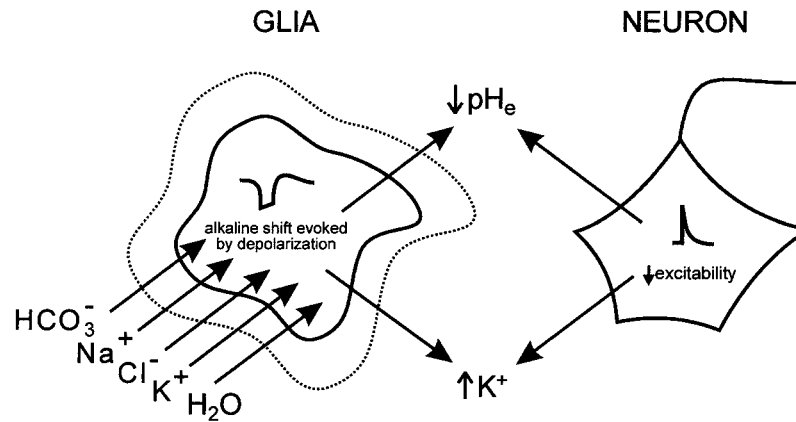


Fig. 3. Schematic of the mechanism of nonspecific feedback suppressing neuronal excitability. Active neurons release K^+ which accumulates in the ECS and depolarizes glial cells. This causes an alkaline shift in glial pH_i and an acid shift in pH_e . Extracellular acidosis further suppresses neuronal activity. Transmembrane ionic movements result in glial swelling, ECS volume decrease and therefore in the greater accumulation of ions and neuroactive substances in the ECS.

cells are immature, or during pathological states when glia becomes reactive (18–20). Figure 3 shows that a nonspecific feedback mechanism suppressing neuronal activity exists in the CNS, involving the following steps:

1. Neuronal activity results in the accumulation of $[K^+]_e$;
2. K^+ depolarizes glial cells, and this depolarization induces an alkaline shift in the glial cytoplasm;
3. The glial cells, therefore, extrude acid;
4. The acid shift in pH_e decreases the neuronal excitability;
5. Glial cells swell, because the ionic changes are always accompanied by water shifts;
6. The ECS shows a compensatory shrinkage, and this results in a greater accumulation of ions and other neuroactive substances during repeated stimulation and subsequently in a further suppression of neuronal activity (4).

Other important chemical components of the ECS are substances involved in metabolism, particularly glucose and dissolved gasses (O_2 and CO_2). The presence of HCO_3^- and CO_2 forms a powerful buffering system which controls extracellular and intracellular pH. The ECS also contains free radical scavengers such as ascorbate and glutathione, which may counteract some potentially lethal products of oxygen metabolism. In addition, the ECS contains amino acids like glutamate and aspartate, catecholamines, indolamines such as dopamine and serotonin, various opioid peptides, nitric oxide (NO) and growth hormones. Transmitters in the ECS can bind to extrasynaptically located high affinity binding sites on neurons and glia.

The solution in the ECS is not a simple salt solution. It has become apparent that long-chain polyelectrolytes, either attached or unattached to cellular membranes, are present in ECS. The ECS also contains a number of glycosaminoglycans (e.g., hyaluronate), glycoproteins, and proteoglycans that constitute the extracellular matrix (ECM). Various ECM molecules and adhesion molecules have also been described,

e.g., fibronectin, tanescin, laminin, and so on, (21,22), the content of which can dynamically change during development, aging, wound healing, and many pathological processes. ECM molecules are produced by both neurons and glia. These molecules have been suggested to cordon off distinct functional units in the CNS (groups of neurons, axon tracts, and nuclear groups). As shown in figure 1, these large molecules can slow down the movement (diffusion) of various neuroactive substances through the ECS. More importantly, these molecules can hinder the diffusion of molecules so that they are confined to certain places, whereas the diffusion to other brain regions will be facilitated.

Glial cells maintain not only ECS ionic homeostasis, but also ECS volume homeostasis (by swelling and shrinking during ionic shifts); they produce various extracellular matrix molecules, and when hypertrophied or proliferating form diffusion barriers (4). In this way glial cells can critically affect the permissiveness of the tissue, synaptic as well as extrasynaptic transmission, activity-dependent synaptic plasticity, neurogenesis, and regeneration.

3. ECS VOLUME AND GEOMETRY: DIFFUSION PARAMETERS

The diffusion of substances released from neurons or glia into the narrow ECS is hindered by the size of the channels between cells, the presence of membranes, fine processes, macromolecules of the extracellular matrix, charged molecules, and also the cellular uptake or degradation of these substances by enzymes (Fig. 1). Diffusion in the ECS obeys Fick's law, albeit subject to important modifications. First, diffusion is constrained by the restricted volume of the tissue available for diffusing particles, i.e., by the extracellular space volume fraction (α), which is the ratio between the volume of the ECS and total tissue volume. It is now clear that the ECS in adult brain amounts to about 20% of the total brain volume, i.e. $\alpha = 0.2$. The second modification to Fick's law is that the free diffusion coefficient (D) in the brain is reduced by the tortuosity factor (λ). ECS tortuosity is defined as $\lambda = (D/ADC)^{0.5}$, where ADC is the apparent diffusion coefficient in the brain and D is a free diffusion coefficient. As a result of tortuosity, D is reduced to an apparent diffusion coefficient $ADC = D/\lambda^2$. Thus, any substance diffusing in the ECS is hindered by membrane obstructions, glycoproteins, macromolecules of the ECM, charged molecules, and fine neuronal and glial cell processes. Third, substances released into the ECS are transported across membranes by nonspecific concentration-dependent uptake (k'). In many cases however, these substances are transported by energy-dependent uptake systems that obey nonlinear kinetics (23). When these three factors (α , λ and k') are incorporated into Fick's law, diffusion in the CNS is described fairly satisfactorily (24). To describe the diffusion constraints on neuroactive substances and their dynamic changes in vivo, the diffusion parameters of the ECS are studied using the real-time iontophoretic method (4,5). This method allows us to follow the diffusion of extracellular markers applied by iontophoresis (Fig. 4). The absolute values of the ECS volume, ADC s, tortuosity as well as nonspecific cellular uptake can be obtained both in vivo or in vitro, during physiological as well as in pathological states. These three parameters are extracted by a nonlinear curve-fitting simplex algorithm operating on the diffusion curve described by Equation [1] below, which represents the behavior of TMA^+ , assuming that it spreads out with spherical symmetry, when the iontophoresis current is applied for duration S .

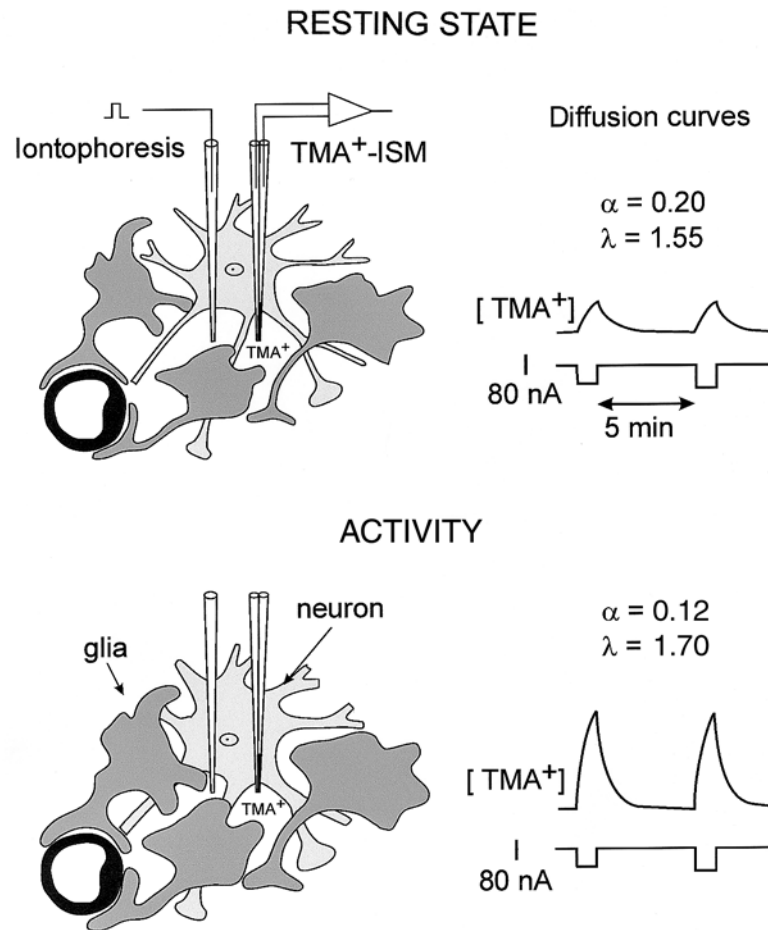


Fig. 4. Experimental set-up, TMA⁺ diffusion curves and typical ECS diffusion parameters α (volume fraction) and λ (tortuosity) in the CNS. Left: Schema of the experimental arrangement. A TMA⁺ selective double-barrelled ion-selective microelectrode (ISM) was glued to a bent iontophoresis microelectrode. The separation between electrode tips was 130–200 μm . Right: Typical TMA⁺ diffusion curves obtained in unstimulated brain (resting state) and during stimulation (activity), evoked by the same iontophoretic current of 80 nA. ECS in unstimulated brain is 20% (volume fraction $\alpha = 0.20$) and tortuosity is about 1.55. When the ECS is smaller due to cell swelling during activity, the diffusion curves are bigger. ECS volume may decrease to about 12% ($\alpha = 0.12$) although tortuosity increases ($\lambda = 1.70$).

In this expression, C is the concentration of the ion at time t and distance r . The equation governing the diffusion in brain tissue is:

$$C = G(t) \quad t < S, \text{ for the rising phase of the curve}$$

$$C = G(t) - G(t - S) \quad t < S, \text{ for the falling phase of the curve.}$$

The function $G(u)$ is evaluated by substituting t or $t - S$ for u in the following equation (24):

$$G(u) = (Q\lambda^2/8\pi D\alpha r) \{ \exp[r\lambda(k'/D)^{1/2}] \operatorname{erfc} [r\lambda/2(Du)^{1/2} + (k'u)^{1/2}] + \exp[-r\lambda(k'/D)^{1/2}] \operatorname{erfc}[r\lambda/2(Du)^{1/2} - (k'u)^{1/2}] \} \quad [1]$$

The quantity of TMA⁺ or TEA⁺ delivered to the tissue per second is $Q = In/zF$, where I is the step increase in current applied to the iontophoresis electrode, n is the transport number, z is the number of charges associated with the substance iontophored (+ for TMA⁺ or TEA⁺), and F is Faraday's electrochemical equivalent. The function "erfc" is the complementary error function. When the experimental medium is agar, by definition, $\alpha=1=\lambda$ and $k' = 0$, and the parameters n and D are extracted by the curve fitting. Knowing n and D , the parameters α , λ , and k' can be obtained when the experiment is repeated in neural tissue.

The other methods used so far to study ECS volume and geometry in vivo have been less comprehensive, since either they can only give information about relative changes in ECS volume, or these changes are only partly related to cell swelling and ECS shrinkage and some other, often unknown mechanisms, can contribute to these signals. These methods include light scattering (25), measurements of tissue resistance (26–28), measurement of the ADCs of molecules tagged with fluorescent dye and followed by optical imaging (29) or changes in the ADC of water (ADC_W) measured by diffusion-weighted NMR (30–33). The optical methods, light reflectance and light transmittance, used in vitro, particularly on brain slices, are believed to reflect changes in ECS volume, however direct evidence is missing. Recently, we found that changes in ECS volume in spinal cord slices measured by the "TMA⁺ method" have a different time course than those revealed by light scattering (Syková et al., unpublished data). Diffusion-weighted NMR methods give information about the water diffusion coefficient, but the relationship between water movement, diffusion ADC_W maps and the changes in cell volume and ECS diffusion parameters (ECS volume fraction and tortuosity) is not yet well understood.

Neuroactive substances released constantly into the ECS will accumulate in this limited volume more rapidly than in free solution. Tortuosity (which is absent in a free medium) also causes a greater and more rapid accumulation of released substances. CNS tortuosity reduces the diffusion coefficient for small molecules by a factor of about 2.5 in many CNS regions. Larger molecules (with a relative molecular mass above 10 kDa), have a smaller diffusion coefficient than small molecules and are significantly more hindered in their diffusion, and therefore exhibit larger tortuosity (34,35). Even large proteins, e.g., negatively charged globular proteins such as bovine serum albumin (66 kDa), or dextrans of 70 kDa, still migrate through the narrow interstices of brain slices (29,36). Recently, the diffusion properties of two types of rather large copolymers of N-(2-hydroxypropyl)methacrylamide (HPMA), developed as water-soluble anti-cancer drug carriers, were studied in rat cortical slices—long-chain HPMA with $M_r = 220$ kDa and globular (bulky) HPMA, containing either albumin (179 kDa) or immunoglobulin (IgG) (319 kDa) in the center with HPMA side branches. Using the integrative optical imaging method and pressure microinjection of fluorescein-tagged polymers, the apparent diffusion coefficients (ADC) were obtained in rat cortical slices (37). The tortuosity for long-chain HPMA was found to be smaller than the tortuosity for globular copolymers (38). These data show that rather than M_r , the shape of the substance is the limiting factor in its movement through the extracellular space.

4. DIFFUSION INHOMOGENEITY AND ANISOTROPY

ECS diffusion parameters are different in different parts of the CNS. For example, it has been recognized that the TMA⁺ diffusion parameters in the sensorimotor cortex of young adult rats *in vivo* are heterogeneous (39). The mean volume fraction gradually increases from $\alpha = 0.19$ in cortical layer II to $\alpha = 0.23$ in cortical layer VI. These typical differences are apparent in each individual animal. In subcortical white matter (corpus callosum) the volume fraction is always lower than in cortical layer VI, often between 0.19–0.20 (40). The mean tortuosity values are typically in the range of 1.51–1.65, and k' values vary between $3.3\text{--}6.3 \times 10^{-3} \text{ s}^{-1}$. There is also a heterogeneity in the spinal cord, the mean values of the volume fraction being highest in the ventral horn ($\alpha = 0.23$) and lowest in the white matter ($\alpha = 0.18$) (41–43). Similar α values ($\alpha = 0.21\text{--}0.22$) have been found throughout the rat hippocampus *in vivo* (44,45), as well as in hippocampal slices (46).

Significant differences in tortuosity have been found in various brain regions, showing that the local architecture is significantly different. There is increasing evidence that diffusion in brain tissue is anisotropic. Isotropy is defined as the state of constant λ in any direction from a point source, whereas anisotropy indicates a difference in λ along different axes. To test for anisotropy, the ECS diffusion parameters are measured in three orthogonal axes x , y and z . Anisotropic diffusion, mediated by the structure of neurons, dendrites, axons and glial processes, can channel the migration of substances in the ECS (preferred diffusion in one direction, e.g., along the axons) and may, therefore, account for a certain degree of specificity in diffusion transmission. Indeed, anisotropic diffusion was described using the “TMA⁺ method” in the white matter of the corpus callosum and spinal cord (40,43) as well as in gray matter in the molecular layer of the cerebellum (47), in hippocampus (44) and in the auditory but not in the somatosensory cortex (48). Using MRI, evidence of anisotropic diffusion in white matter was found in cat brain (49) as well as in human brain (50). Therefore, not only the diffusion of large molecules, such as TMA⁺ or dextrans, but even the diffusion of water, are prevented by the presence of myelin sheaths. Because of the distinct diffusion characteristics, the extracellular molecular traffic will be different in various brain regions. The anisotropy of the white and gray matter could enable different modes of diffusion transmission in these regions.

4.1. Role of Glia in ECS Volume and Geometry Changes

Ions as well as neurotransmitters released into the ECS during neuronal activity or pathological states interact not only with the postsynaptic and presynaptic membranes, but also with extrasynaptic receptors, including those on glial cells. Stimulation of glial cells leads to the activation of ion channels, second messengers and intracellular metabolic pathways, and to changes in glial volume that are accompanied by dynamic variations in ECS volume, particularly the swelling and possible rearrangement of glial processes. In addition to their role in the maintenance of extracellular ionic homeostasis, glial cells may thus, by regulating their volume, influence the extracellular pathways for neuroactive substances.

Many pathological processes in the CNS are accompanied by a loss of cells or neuronal processes, astrogliosis, demyelination, and changes in the extracellular matrix, all of which may affect the apparent diffusion coefficients of neuroactive substances. Sev-

eral animal models have been developed to study changes in ECS diffusion parameters. Brain injury of any kind elicits reactive gliosis, involving both hyperplasia and hypertrophy of astrocytes, which show intense staining for glial fibrillary acidic protein (GFAP) (51). Astroglia is also a typical characteristic of cortical stab wounds in rodents (51). The lesion is typically accompanied by an ECS volume increase and a substantial tortuosity increase to mean values of α of about 0.26 and λ of about 1.77 (Fig. 5A) (52).

Similarly, both the size of the ECS (α) and, surprisingly, also λ , were significantly higher in cortical grafts than in host cortex, about 0.35 and 1.79, respectively, as is also the case in gliotic cortex after stab wounds. Both α and λ were increased in cortical grafts of fetal tissue transplanted to the midbrain, where severe astroglia compared to host cortex was found, but not in fetal grafts placed into a cavity in the cortex, where only mild astroglia occurred (53). Another characteristic feature of cortical grafts into midbrain was the variability of α and λ . The different values found at various depths of the grafts correlated with the morphological heterogeneity of the graft neuropil. These measurements show that even when the ECS in gliotic tissue or in cortical grafts is larger than in normal cortex, the tortuosity is still higher, and the diffusion of chemical signals in such tissue may be hindered. Limited diffusion may also have a negative impact on the viability of grafts in host brains. Compared to host cortex, immunohistochemistry showed myelinated patches and a larger number of hypertrophic astrocytes in areas of high λ values, suggesting that more numerous and/or thicker glial cell processes might be a cause of the increased tortuosity.

Cell swelling and astroglia (manifested as an increase in GFAP) were also evoked in isolated rat spinal cords of 4–21-d-old rats by incubation in either 50 mM K^+ or hypotonic solution (235 mosmol kg^{-1}). Application of K^+ and hypotonic solution resulted at first in a decrease in the ECS volume fraction and in an increase in tortuosity in spinal gray matter (Fig. 5B). These changes resulted from cell swelling, since the total water content (TW) in spinal cord was unchanged and the changes were blocked in Cl^- -free solution and slowed down by furosemide and bumetanide. During a continuous 45 min application, α and λ started to return toward control values, apparently due to the shrinkage of previously swollen cells since TW remained unchanged. This return was blocked by fluoroacetate, suggesting that most of the changes were due to the swelling of glia. A 45 min application of 50 mM K^+ and, to a lesser degree, of hypotonic solution evoked astroglia, which persisted after washing out these solutions with physiological saline. During astroglia λ increased again to values as high as 2.0, whereas α either returned to or increased above control values. This persistent increase in λ after washout was also found in white matter. These data show that glial swelling and astroglia are associated with a persistent increase in ECS diffusion barriers.

4.2. Diffusion Parameters and Extracellular Matrix

ECM molecules and other large molecules can also affect the tortuosity of the ECS. Their possible effect on changes in TMA^+ diffusion parameters has been studied in rat cortical slices (34,36,37) and in isolated rat spinal cord (54). Superfusion of the slice or spinal cord with a solution containing either 40-kDa or 70-kDa dextran or hyaluronic acid (HA) resulted in a significant increase in λ . In standard physiological solution, λ was about 1.57, whereas in a 1% or 2% solution of 40-kDa or 70-kDa dex-

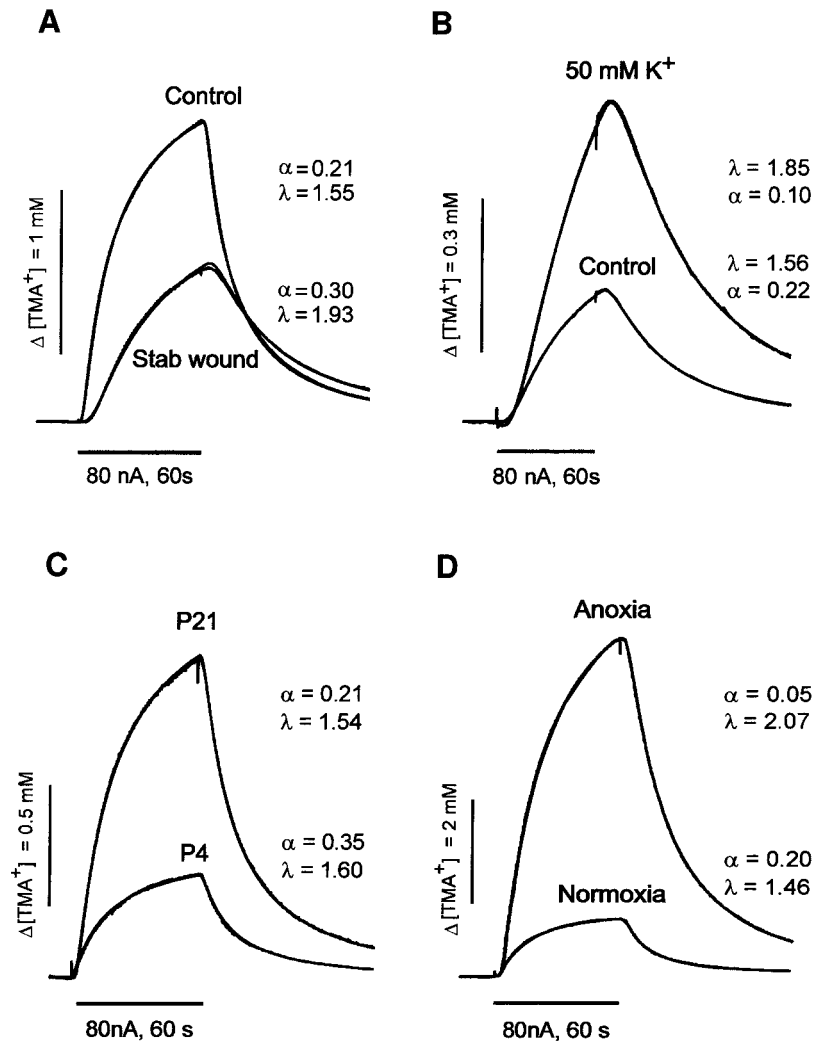


Fig. 5. TMA⁺ diffusion curves under different experimental conditions. For each curve, the ECS diffusion parameters α (volume fraction) and λ (tortuosity) were extracted by appropriate nonlinear curve fitting. Experimental and theoretical curves are superimposed in each case. For each figure the concentration scale is linear. **(A)** Typical recordings obtained in rat cortex (control). Values of α and λ are increased in the gliotic cortex around a stab wound. Note that the larger the curve, the smaller the value of α ; a slower rise and decay indicate higher tortuosity. **(B)** Typical recordings in isolated rat spinal cord of a 13-d-old rat before (control) and during application of 50 mM K⁺. Cellular, particularly glial, swelling results in an ECS volume decrease and a tortuosity increase. **(C)** Typical recordings obtained in rat cortex at postnatal days (P) 4 and 21. Note the dramatic decrease in the ECS volume and the increased tortuosity during maturation. **(D)** Typical recordings obtained in adult rat cortex (lamina V) during normoxia and in the same animal about 10 min after cardiac arrest (anoxia).

tran, λ increased to about 1.72–1.77. Application of a 0.1% solution of HA (1.6×10^6 Da) resulted in an increase in λ to about 1.75. The α was unchanged in these experiments, suggesting that these substances had no effect on cell volume and the viability of the preparation.

A decrease in tortuosity and a loss of anisotropy have also been found during aging. This decrease correlates with the disappearance of fibronectin and chondroitin sulphate proteoglycans, forming perineuronal nets around granular and pyramidal cells in the hippocampus of young adult rats. These results suggest that bigger molecules such as 40- and 70-kDa dextran, hyaluronic acid, and molecules of the extracellular matrix may slow down the diffusion of small molecules such as TMA⁺ (74 Da) in the ECS.

5. ECS DIFFUSION PARAMETERS DURING DEVELOPMENT

Compared to healthy adults, ECS diffusion parameters significantly differ during postnatal development (39,40,43,55). The ECS volume in the cortex is about twice as large ($\alpha = 0.36$ – 0.46) in the newborn rat as in the adult rat ($\alpha = 0.21$ – 0.23), while the tortuosity increases with age (Fig. 5C). The reduction in the ECS volume fraction correlates well with the growth of blood vessels. In rat spinal cord gray matter, α decreases with neuronal development and gliogenesis from postnatal day 4 to 12 by about 15%, while λ significantly increases, showing that the diffusion of molecules becomes more hindered with age. The large ECS channels during development may allow the migration of larger substances (e.g., growth factors) and better conditions for cell migration during development. On the other hand, the large ECS in the neonatal brain could significantly dilute ions, metabolites, and neuroactive substances released from cells, relative to release in adults, and may be a factor in the prevention of anoxic injury, seizure and spreading depression in young individuals. The diffusion parameters could also play an important role in the developmental process itself. Diffusion parameters are substantially different in myelinated and unmyelinated white matter (40,43). Isotropic diffusion was found in corpus callosum and spinal cord white matter of young rats with incomplete myelination. In myelinated spinal cord and corpus callosum, the tortuosity is higher (the apparent diffusion coefficient is lower) when TMA⁺ diffuses across the axons than when it diffuses along the fibers (Fig. 6).

6. ECS IN PATHOLOGICAL STATES

Pathological states, e.g., anoxia/ischemia, are accompanied by a lack of energy, seizure activity, the excessive release of transmitters and neuroactive substances, neuronal death, glial cell loss or proliferation, glial swelling, the production of damaging metabolites including free radicals and the loss of ionic homeostasis. Others are characterized by inflammation, edema or demyelination. It is therefore evident that they will be accompanied not only by substantial changes in ECS ionic composition (1,3) but also by various changes in ECS diffusion parameters according to the different functional and anatomical changes.

5.1. ECS Ionic Composition and Volume during Anoxia/Ischemia

Dramatic K⁺ and pH_e changes occur in the brain and spinal cord during anoxia and/or ischemia (3,41,56). Within 2 min after respiratory arrest in adult rats, blood

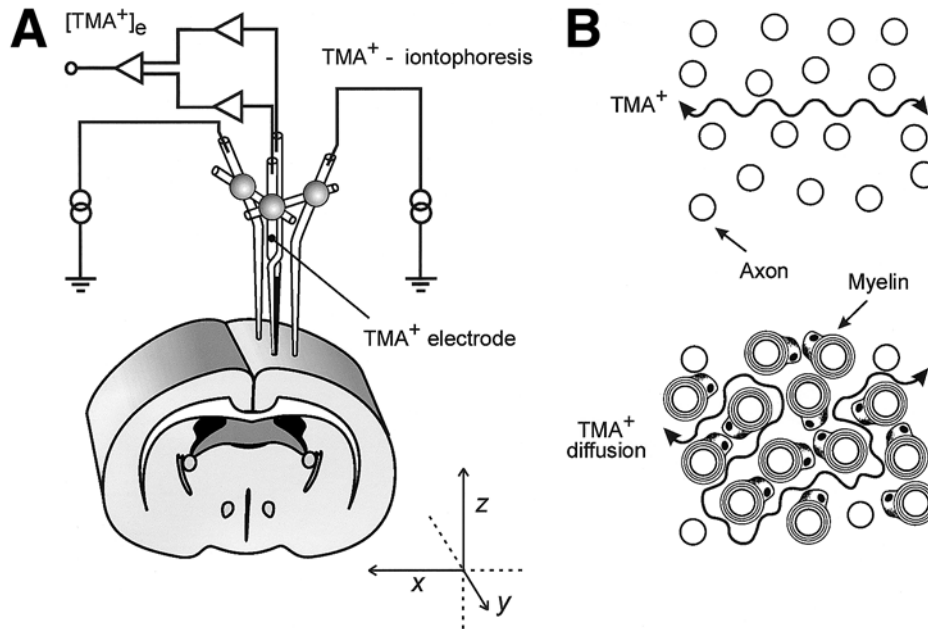


Fig. 6. (A) Schema of experimental arrangement to study diffusion anisotropy. A TMA⁺ selective double-barrelled ion-selective microelectrode (ISM) was glued to two bent iontophoresis microelectrodes. Separation between electrode tips was 110–180 μm . Tips of the 3 pipets formed a 90° horizontal angle for simultaneous measurements along the x - and y -axes. Similarly, for measurements along the z -axis, 1 iontophoresis pipet tip was lowered 110–180 μm below the tip of the ISM. (B) Extracellular diffusion in the direction perpendicular to the orientation of the axons, i.e., around axons, is compromised by the number of myelin sheaths, the number of myelinated axons, and the length of the myelin sheaths along axons. The scheme demonstrates the increased anisotropy as myelination progresses.

pressure begins to increase and pH_e begins to decrease (by about 0.1 pH unit), although $[\text{K}^+]_e$ remains unchanged. With the subsequent blood pressure decrease, the pH_e decreases by 0.6–0.8 pH units to pH 6.4–6.6. This pH_e decrease is accompanied by a steep rise in $[\text{K}^+]_e$ to about 50–70 mM; decreases in $[\text{Na}^+]_e$ to 48–59 mM, $[\text{Cl}^-]_e$ to 70–75 mM, $[\text{Ca}^{2+}]_e$ to 0.06–0.08 mM, and pH_e to 6.1–6.8; an accumulation of excitatory amino acids; a negative DC slow potential shift; and a decrease in ECS volume fraction to 0.04–0.07. The ECS volume starts to decrease when the blood pressure drops below 80 mm Hg and $[\text{K}^+]_e$ rises above 6 mM (41).

Figure 5D shows that during hypoxia and terminal anoxia, the ECS volume fraction in rat cortex or spinal cord decreases from about 0.20 to about 0.05, although tortuosity increases from 1.5 to about 2.1 (41,57). The same ultimate changes were found in all neonatal, adult and aged rats, in grey and white matter, in the cortex, corpus callosum and spinal cord. However, the time course in white matter was significantly slower than in gray matter; and the time course in neonatal rats was about 10 times slower than in adults (55). Linear regression analysis revealed a positive correlation between the normoxic size of the ECS volume and the time course of the changes. This corresponds to

the well-known resistance of the immature CNS and the greater susceptibility of the aged brain to anoxia.

5.2. Changes in Extracellular Space Volume and Geometry during X-irradiation Injury

Radiation therapy is an effective treatment for some human cancers. However, this therapy is limited by radiation injury. Clinical correlates of demyelination and necrosis, which occur early, include somnolence, changes in intellect, radiation myelopathies, and leucoencephalopathies. The responses of the normal tissue immediately surrounding the tumor to its unavoidable irradiation are therefore of considerable interest. It is generally accepted that radiation injury is caused by mitotic death and the depletion of the various cell populations. The main symptoms of radiation injury in the CNS are very similar in rodents and humans. The immature CNS is more sensitive to radiation than the adult nervous system, apparently due to the proliferative potential and increased radiation sensitivity of glial and/or vascular endothelial cells in the developing nervous system (58).

In experiments on the somatosensory neocortex and subcortical white matter of one-day-old (P1) rats, X-irradiation with a single dose of 40 Gy resulted in radiation necrosis with typical early morphological changes in the tissue, namely cell death, DNA fragmentation, extensive neuronal loss, blood-brain-barrier (BBB) damage, activated macrophages, astrogliosis, an increase in extracellular fibronectin, and concomitant changes in all three diffusion parameters. The changes were observed as early as 48 h postirradiation and persisted at P21 (59).

In the nonirradiated cortex, the volume fraction, α , of the ECS is large in newborn rats and diminishes with age (39). X-irradiation with a single dose of 40 Gy blocked the normal pattern of volume fraction decrease during postnatal development and, in fact, brought about a significant increase (59). At P4–P5, α in both cortex and corpus callosum increased to about 0.5. The large increase in α persisted at 3 wk after X-irradiation. Tortuosity, λ , and nonspecific uptake, k' , significantly decreased at P2–P5; at P8–P9 they were not significantly different from those of control animals, although they significantly increased at P10–P21. This means that in chronic lesions, e.g., those occurring 1–3 wk after X-irradiation and/or in gliotic tissue, the volume fraction remains elevated, but tortuosity increases. Interestingly, X-irradiation with a single dose of 20 Gy, which resulted in relatively light neuronal damage and loss and BBB damage, did not produce changes in diffusion parameters significantly different from those found with 40 Gy. Less pronounced but still significant changes in diffusion parameters were also found in areas of the ipsilateral and contralateral hemispheres adjacent to the directly X-irradiated cortex (59).

The observed increase in the extracellular volume fraction in the X-irradiated tissue could also contribute to the impairment of signal transmission frequently observed after X-irradiation employed in the clinical treatment of various tumors, e.g., by diluting ions and neuroactive substances released from cells, and may play an important role in functional deficits as well as in the impairment of the developmental processes. Moreover, the increase in tortuosity, inferred from the decrease in the ADC_{TMA} , in the X-irradiated cortex and in the contralateral hemisphere, suggests that even long after mild irradiation the diffusion of substances can be substantially hindered. The observed

increase in tortuosity seems to be related to astrogliosis, but changes in adhesion molecules or extracellular matrix molecules could also account for it.

5.3. ECS Volume and Geometry During Inflammation and Demyelination

Changes in ECS diffusion parameters can be expected during inflammation during which brain edema may develop. In an experimental model, inflammation was evoked by an intracerebral inoculation of a weakly pathogenic strain of *Staphylococcus aureus* (60). Acute inflammation and an increase in BBB permeability in the abscess region resulted in rather mild changes in the ECS diffusion parameters, i.e., volume fraction tended to be somewhat larger and the tortuosity somewhat smaller.

Dramatic changes in the ECS diffusion parameters were found in the spinal cord of rats during experimental autoimmune encephalomyelitis (EAE), an experimental model of multiple sclerosis (42). EAE, which was induced by the injection of guinea-pig myelin basic protein (MBP), resulted in typical morphological changes in the CNS tissue, namely demyelination, an inflammatory reaction, astrogliosis, BBB damage, and paraparesis, at 14–17 d postinjection. Paraparesis was accompanied by increases in α in the dorsal horn, in the intermediate region, in the ventral horn and in white matter from about 0.18 to about 0.30. There were significant decreases in λ in the dorsal horn and the intermediate region and decreases in k' in the intermediate region and the ventral horn (42). There was a close correlation between the changes in ECS diffusion parameters and the manifestation of neurological abnormalities.

These results suggest that the expansion of the ECS alters diffusion parameters in inflammatory and demyelinating diseases and may affect the accumulation and movement of ions, neurotransmitters, neuromodulators and metabolites in the CNS in these disorders, possibly by interfering with axonal conduction.

6. ECS DIFFUSION PARAMETERS AND AGING

Aging, Alzheimer's disease, and many degenerative diseases are accompanied by serious cognitive deficits, particularly impaired learning and memory loss. This decline in old age is a consequence of changes in brain anatomy, morphology, volume, and functional deficits. Nervous tissue, particularly in the hippocampus and cortex, is subject to various degenerative processes including a decreased number and efficacy of synapses, a decrease in transmitter release, neuronal loss, astrogliosis, demyelination, deposits of beta amyloid, changes in extracellular matrix proteins, and so on. These and other changes not only affect the efficacy of signal transmission at synapses, but could also affect extrasynaptic ("volume") transmission mediated by the diffusion of transmitters as well as other substances through the volume of the extracellular space (ECS). Many mediators, including glutamate and GABA, bind to high affinity binding sites located at nonsynaptic parts of the membranes of neurones and glia. Mediators that escape from the synaptic clefts at an activated synapse, particularly following repetitive stimulation, diffuse in the ECS and can crossreact with receptors in nearby synapses. This phenomenon, called "cross-talk" between synapses by the "spillover" of a transmitter (e.g., glutamate, GABA, glycine), has been proposed to account for LTP and LTD in the rat hippocampus (61,62). The cross-talk between synapses, and the efficacy and directionality of volume transmission, could be critically dependent on the diffusion properties of the ECS.

The ECS diffusion parameters α , $\lambda_{x,y,z}$ and k' were measured in the cortex, corpus callosum, and hippocampus (CA1, CA3 and in dentate gyrus). If diffusion in a particular brain region is anisotropic, then the correct value of the ECS volume fraction cannot be calculated from measurements done only in one direction. For anisotropic diffusion, the diagonal components of the tortuosity tensor are not equal, and generally its nondiagonal components need not be zero. Nevertheless, if a suitable referential frame is chosen (i.e., if we measure in three privileged orthogonal directions), neglecting the nondiagonal components becomes possible, and the correct value of the ECS size can thus be determined (44,47). Therefore, TMA⁺ diffusion was measured in the ECS independently along three orthogonal axes (x – transversal, y – sagittal, z – vertical). In all three regions—cortex, corpus callosum and hippocampus—the mean ECS volume fraction α was significantly lower in aged rats (26–32-mo-old), ranging from 0.17 to 0.19, than in young adults (3–4-mo-old) in which α ranged from 0.21 to 0.23 (Fig. 7). Nonspecific uptake k' was also significantly lower in aged rats. From Fig. 7 it is evident that the diffusion curves for the hippocampus are larger in the aged rat than in the young one, i.e., the space available for TMA⁺ diffusion is smaller. Although the mean tortuosity values along the x -axis are not significantly different between young and aged rats, the values are significantly lower in aged rats along the y - and z -axes (Fig. 7), and thus the values along all three axes become the same. This means that there is a loss of anisotropy in the aging hippocampus, particularly in the CA3 region and the dentate gyrus (45).

The three-dimensional pattern of diffusion away from a point source can be illustrated by constructing iso-concentration spheres (isotropic diffusion) or ellipsoids (anisotropic diffusion) for extracellular TMA⁺ concentration. The surfaces in figure 7 represent the locations where TMA⁺ concentration first reached 1 mM, 60s after its application in the center. The ellipsoid in the hippocampus of the young adult rat reflects the different abilities of substances to diffuse along the x -, y - and z -axes, whereas the sphere in the hippocampus of the aged rat shows isotropic diffusion (Fig. 7). The smaller ECS volume fraction in aged rats is reflected in the sphere's being larger than the ellipsoid.

Morphological changes during aging include cell loss, loss of dendritic processes, demyelination, astrogliosis, swollen astrocytic processes, and changes in the extracellular matrix. It is reasonable to assume that there is a significant decrease in the *ADC* of many neuroactive substances in the aging brain, which accompanies astrogliosis and changes in the extracellular matrix. One of the explanations why α in the cortex, corpus callosum, and hippocampus of senescent rats is significantly lower than in young adults could be astrogliosis in the aged brain. Increased GFAP staining and an increase in the size and fibrous character of astrocytes have been found in the cortex, corpus callosum and hippocampus of senescent rats, which may account for changes in the ECS volume fraction (45). Other changes could account for the decreases in λ values and for the disruption of tissue anisotropy. In the hippocampus in CA1, CA3, as well as in the dentate gyrus, we observed changes in the arrangement of fine astrocytic processes. These are normally organized in parallel in the x - y plane (Fig. 8A,B), and this organization totally disappears during aging. Moreover, the decreased staining for chondroitin sulfate proteoglycans and for fibronectin (Fig. 8C,D) suggests a loss of extracellular matrix macromolecules.

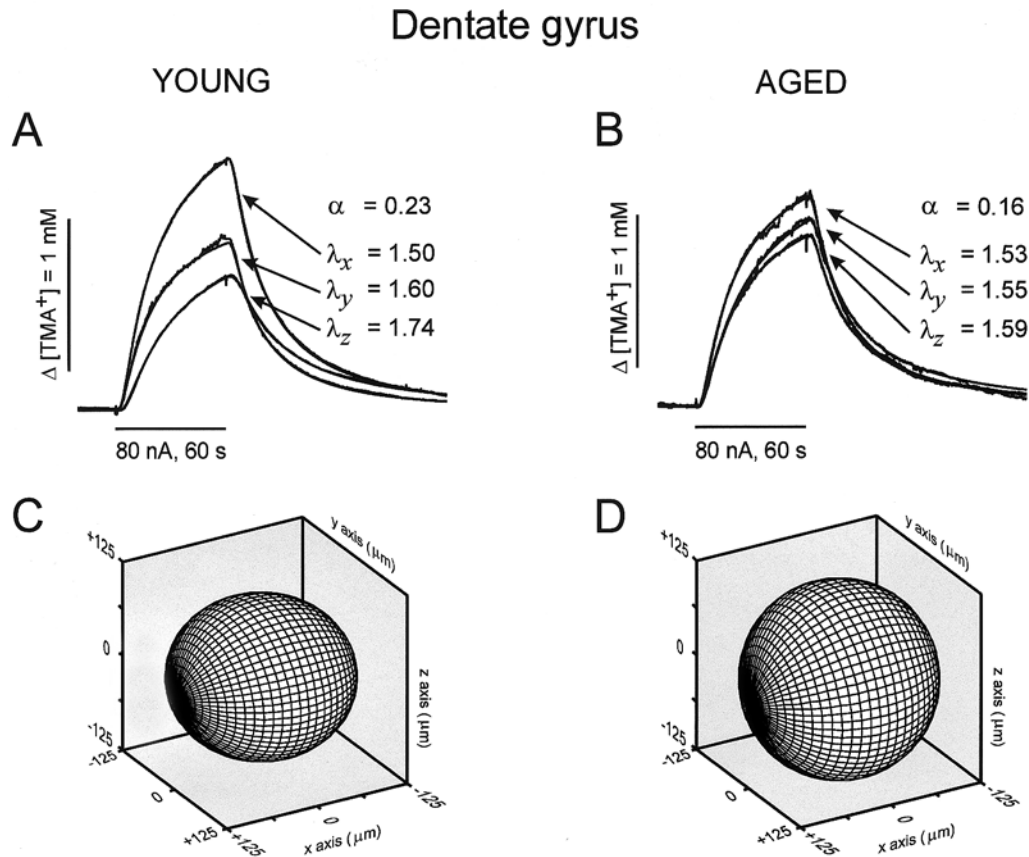


Fig. 7. Diffusion parameters in the hippocampus dentate gyrus of a young adult and an aged rat. **(A)** Anisotropic diffusion in the dentate gyrus of a young adult rat. TMA⁺ diffusion curves (concentration-time profiles) were measured along three orthogonal axes (*x*-mediolateral, *y*-rostrocaudal, *z*-dorsoventral). The slower rise in the *z* than in the *y* direction and in the *y* than in the *x* direction indicates a higher tortuosity and more restricted diffusion. The amplitude of the curves shows that TMA⁺ concentration, at approximately the same distance from the tip of the iontophoresis electrode, is much higher along the *x*-axis than along the *y*-axis and even higher than along the *z*-axis (λ_x , λ_y , λ_z). This can be explained if we realize that TMA⁺ concentration decreases with the “diffusion distance” from the iontophoretic micropipet and that the real “diffusion distance” is not *r* but λr . Note that the actual ECS volume fraction α is about 0.2 and can be calculated only when measurements are done along the *x*-, *y*- and *z*-axes. **(B)** Anisotropy is almost lost in an aged rat; diffusion curves are higher, showing that α is smaller. **C** and **D**: Iso-concentration surfaces for a 1.0 mM TMA⁺ concentration contour 60s after the onset of a 80 nA iontophoretic pulse. The surfaces were generated using mean values of volume fraction and tortuosity. **(C)** The ellipsoid represent anisotropic diffusion in a young adult rat. **(D)** The larger sphere in an aged rat, corresponding to isotropic diffusion and to a lower ECS volume fraction, demonstrates that diffusion from any given source will lead to a higher concentration of substances in the surrounding tissue and a larger action radius in aged rats than in young adults.

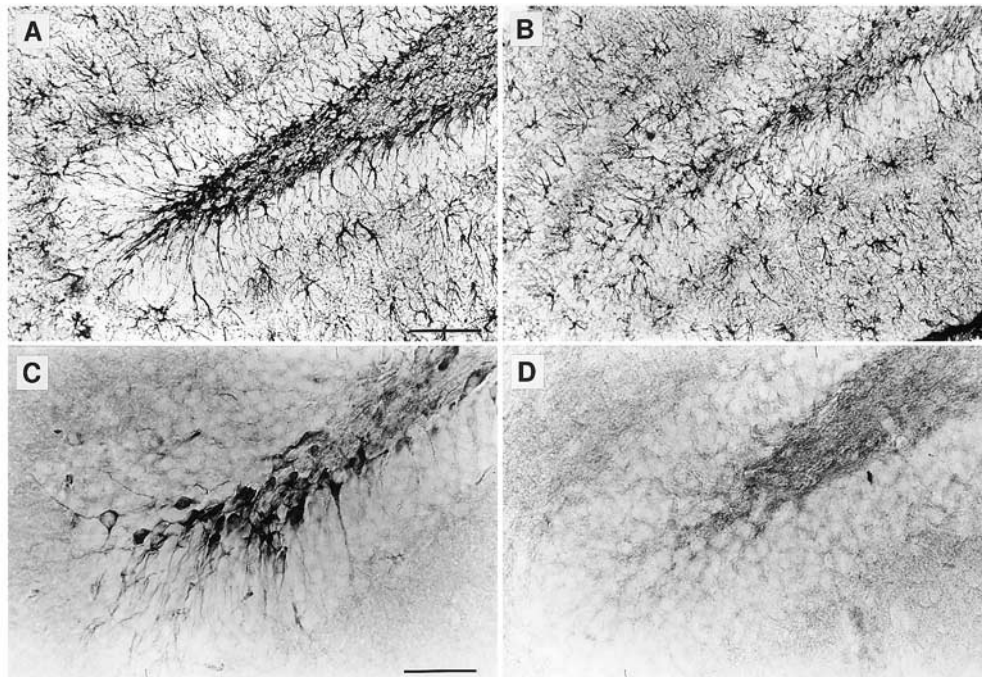


Fig. 8. Structural changes in the hippocampus gyrus dentatus region of aged rats. **(A)** Astrocytes stained for GFAP in a young adult rat; note the radial organization of the astrocytic processes between the pyramidal cells (not stained). **(B)** In an aged rat the radial organization of the astrocytic processes is lost. **(C)** Staining for fibronectin in a young adult rat shows densely stained cells, apparently due to perineuronal staining around granular cells. **(D)** In an aged rat the fibronectin staining is lost. Scale bar: in **A** and **B** = 100 μm , **C** and **D** = 50 μm .

Because α is lower in aging rats, we expected some differences in the ECS diffusion parameter changes during ischemia in senescent rats. Our study revealed that the final values of α , λ and k' induced by cardiac arrest are not significantly different between young and aged rats; however, the time course of all the changes is faster in aged animals (45). The first peak in TMA⁺ baseline changes, indicating an ECS volume decrease and a K⁺ increase, was reached in young adult rats at about 3 min, whereas in senescent rats the time to peak was significantly shorter, about 1.5 min. The second and final peak in the TMA⁺ baseline, associated with an extracellular acid shift, was reached in young adult rats at about 20 min, although in senescent rats this time to peak was again significantly shorter, about 12 min. The faster changes in extracellular space volume fraction and tortuosity in nervous tissue during aging can contribute to a faster impairment of signal transmission, e.g., because of a faster accumulation of ions and neuroactive substances released from cells and their slower diffusion away from the hypoxic/ischemic area in the more compacted ECS.

Our recent study also revealed that the degree of learning deficit during aging correlates with the changes in ECS volume, tortuosity and nonspecific uptake (63). The hippocampus is well-known for its role in memory formation, especially the declarative memory. It is therefore reasonable to assume that diffusion anisotropy, which leads to a certain degree of specificity in extrasynaptic communication, may play an important

role in memory formation. There was a significant difference between mildly and severely behaviorally impaired rats (rats were tested in a Morris water maze), which was particularly apparent in the hippocampus. The ECS in the dentate gyrus of severely impaired rats was significantly smaller than in mildly impaired rats. Also, anisotropy in the hippocampus of severely impaired rats, particularly in the dentate gyrus, was much reduced, although a substantial degree of anisotropy was still present in aged rats with a better learning performance. Anisotropy might be important for extrasynaptic transmission by channeling the flux of substances in a preferential direction. Its loss may severely disrupt extrasynaptic communication in the CNS, which has been suggested to play an important role in memory formation (5,45).

Volume fraction is thus decreasing during the entire postnatal life with the steepest decrease in early postnatal development (39,40). The larger ECS (30–45%) in the first days of postnatal development in the rat can be attributed to incomplete neuronal migration, gliogenesis, angiogenesis, and to the presence of large extracellular matrix proteoglycans, particularly hyaluronic acid, which because of the mutual repulsion of its highly negatively charged branches, occupies a great deal of space and holds cells apart from each other. The ensuing decrease in the ECS size could be explained by the disappearance of a significant part of the ECS matrix, neuron migration, and the development of dendritic trees, rapid myelination and proliferation of glia. Some of these processes are also observed during aging. The most important are probably neuronal degeneration, a further loss of extracellular matrix and astrogliosis. Indeed, we observed a decrease of fibronectin and chondroitin sulfate proteoglycan staining in the hippocampus of mildly impaired aged rats and almost a complete loss of staining in severely impaired aged rats. Chondroitin sulfate proteoglycans participate in multiple cellular processes (64,65). These include axonal outgrowth, axonal branching and synaptogenesis, which are important for the formation of memory traces.

One of the most striking changes we observed was the reduction of nonspecific TMA⁺ uptake in aged rats. The underlying processes for TMA⁺ uptake are so far unknown. These may include transfer into cells or binding to cellular surfaces or to negatively charged molecules of the extracellular matrix. All of these may be reduced during aging. Transfer into cells might decrease due to reduced pinocytosis (stiffer membranes due to a higher proportion of cholesterol), binding to cellular surfaces because of reduced membrane potential and binding to the extracellular matrix because of its loss. It is also interesting that uptake was reduced mainly in cell-body-rich areas and not in the corpus callosum.

What is the functional significance of the observed changes in ECS diffusion parameters during aging? Anisotropy, which, particularly in the hippocampus and corpus callosum, may help to facilitate the diffusion of neurotransmitters and neuromodulators to regions occupied by their high affinity extrasynaptic receptors, might have crucial importance for the specificity of signal transmission. The importance of anisotropy for the “spill-over of glutamate,” “cross-talk” between synapses, and for LTP and LTD has been proposed (61,62). The observed loss of anisotropy in senescent rats could therefore lead to impaired cortical and, particularly, hippocampal function. The decrease in ECS size could be responsible for the greater susceptibility of the aged brain to pathological events (particularly ischemia) (45), the poorer outcome of clinical therapy and the more limited recovery of affected tissue after insult.

7. CONCLUSION

There is increasing evidence that long-term changes in the physical and chemical parameters of the ECS accompany many physiological and pathological states including CNS trauma and aging. The “acute” or relatively fast changes in the size of the intercellular channels are apparently a consequence of cellular (particularly glial) swelling. Abrupt ECS volume decrease may cause “molecular crowding” which can lead to an acute increase in tortuosity. Long-term changes in diffusion would require changes in ECS composition, either permanent changes in the size of the intercellular channels, changes in extracellular matrix molecules, or in the number and thickness of cellular (glial) processes. Available data suggest that in some pathophysiological states α and λ behave as independent variables. A persistent increase in λ (without a decrease in ECS volume) is always found during astrogliosis and in myelinated tissue, suggesting that glial cells can form diffusion barriers, make the nervous tissue less permissive and play an important role in signal transmission, tissue regeneration, and pathological states. This observation has important implications for our understanding of the function of glial cells. The extracellular matrix apparently also contributes to diffusion barriers and to diffusional anisotropy, because its loss, e.g., during aging, correlates with a tortuosity decrease and loss of anisotropy.

In general, changes in ECS ionic composition, size and geometry may affect:

1. Synaptic transmission (width of synaptic clefts, permeability of ionic channels, concentration of transmitters, dendritic length constant, and so on).
2. Extrasynaptic “volume” transmission by diffusion (diffusion of factors such as ions, NO, transmitters, neuropeptides, neurohormones, growth factors and metabolites).
3. Neuronal interaction and synchronization.
4. Neuron-glia communication.
5. ECS ionic homeostasis and glial function.
6. Clearance of metabolites and toxic products.
7. Permeability of ionic channels.
8. Regeneration processes.
9. Long-term changes in local CNS architecture including ECS volume and tortuosity, which apparently can influence plastic changes, LTP or LTD, changes in behaviour and memory formation.

Hopefully, we will more fully understand all of those consequences of an altered ECS in the near future.

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Intercellular Diffusional Coupling between Glial Cells in Slices from the Striatum

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1. INTRODUCTION

In the central nervous system (CNS), macroglial cells (astrocytes and oligodendrocytes) represent the main cell population coupled by gap junctions. Indeed, a much higher level of gap junctional communication is observed in macroglial cells than in neurons and this communication process persists up to the adult stage in these cells (1). Gap junctions are characterized by clusters of intercellular channels connecting the cytoplasm of adjacent cells. These channels are constituted by two hemichannels, the connexons, each of them being formed by six structural subunit proteins, the connexins (Cxs). Cxs are organized around a relatively large hydrated pore which allows the diffusion of ions and small molecules up to 1–1.2 kDa, providing thus the morphological basis for electrical and metabolic coupling (2). Although Cxs are expressed in neurons as well as in glial and ependymal cells (3), glial cells and astrocytes, particularly, contain the highest level of Cxs from the late embryo to the adult (4). Cx43, which is prevalent in astrocytes, is detected early during development, peaks at birth, and remains constant throughout the adult life (4). Cx30 is also found in mature astrocytes and its distribution resembles that of Cx43 (5). Experiments performed with low molecular weight tracers (Lucifer yellow, biocytin, neurobiotin) have indicated that populations of 10 to 100 astrocytes can communicate through gap junction channels in either cortical, hippocampal, or cerebellar slices (6–8). However, although Cx43 was also detected in striatal astrocytes, as reported in a previous study, the intercellular diffusion of the low molecular weight fluorescent dye Lucifer yellow was not detected in astrocytes injected with this dye in striatal slices (9,10). This is surprising because functional studies performed on astrocytic primary cultures from different brain structures, including the striatum, have demonstrated that these cells are highly coupled by gap junction channels (3,11). A marked regional heterogeneity was found in these cell culture studies (12,13) but astrocytes from either the cerebral cortex, the hippocampus, the brain stem, the cerebellum or the striatum were all shown to express Cx43 and to exchange fluorescent dyes, calcium signaling molecules, glucose, and glutamate (14).

Due to the occurrence of cytoplasmic exchanges through gap junction channels, the contribution of astrocytes to several brain functions should not only be considered as resulting from individual elements, but also from groups of communicating and coordinated cells. This is likely to be the case either for intracellular and extracellular ionic homeostasis (15,16), trafficking of metabolic substrates (17) or for the implication of astrocytes in neuronal survival and toxicity (18,19).

In the present study, acute slices were used to determine whether gap junction-mediated intercellular communication occurs between striatal glial cells. Intracellular injections of Lucifer yellow or neurobiotin were performed thanks to the patch-clamp technique using the whole-cell configuration. In agreement with previous findings made on primary cultures, striatal glial cells identified as putative astrocytes were found to be coupled by gap junction channels. In addition, several electrophysiological passive properties of these coupled glial cells were found to be modified by treatments known to inhibit junctional permeability. According to these observations, astrocytes in striatal slices are connected by functional gap junctions and regulations of these intercellular channels may affect the basic electrophysiological properties of these cells.

2. EXPERIMENTAL PROCEDURES

2.1. Slice Preparation

OFA rats (8- to 14-d-old) were decapitated and their brain was quickly removed and stored in cold (4°C) standard bath solution containing (in mM) NaCl 125, KCl 2.5, NaHCO₃ 22, KH₂PO₄ 1.15, 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 5, MgCl₂ 1.5, CaCl₂ 1.5, D-glucose 10, oxygenated with 95% O₂-5% CO₂. Coronal slices from the striatum (300 μm thick) were cut using a microslicer (DTK 1000, DSK). After 45 min at least of recovery in a storage beaker at room temperature (20-25°C), a slice was transferred to the recording chamber mounted on an upright microscope (Axioskop, Zeiss) and perfused with standard bath solution at the rate of 2.5-3 mL per min.

2.2. Electrophysiology

Cells were visualized using differential interference contrast (DIC) microscopy, equipped with a 40X water immersion objective and an infrared-sensitive video camera (C2400, Hamamatsu) in combination with a video graphic printer (UP 890-CE, Sony). Patch-clamp recordings were performed in whole-cell configuration with an Axopatch-ID amplifier (Axon Instruments). Electrodes were filled with a buffer solution containing (in mM): KCl 120, MgCl₂ 2, CaCl₂ 0.5, ethylene glycol-bis (-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) 0.5, HEPES (Na salt) 10, pH adjusted at 7.2 with KOH. The patch pipet resistance was in the range of 3-5 MΩ. The capacity transient minimization and series resistance compensation were performed in response to 10 mV and 10 ms hyperpolarizing voltage steps. After compensation to 50-80%, series resistances ranged from 4 to 9 MΩ. Similar voltage steps were used to determine the cell input membrane resistance. For each depolarizing response, the leak current was determined in response to an hyperpolarizing step of the same amplitude and subtracted off-line. Signals were filtered at 5 kHz, digitized with an ACQUIS1 program (G. Sadok, Paris) for storage and off-line analysis.

2.3. Study of Intercellular Diffusional Coupling

In preliminary experiments, gap junction-mediated coupling was studied by adding 0.1% Lucifer yellow CH (dipotassium salt, Sigma) to the pipet solution. However, most results were obtained using pipets filled with 0.5% neurobiotin (Vector Labs), the KCl concentration of the internal solution being lowered to 105 mM for compensation of the hyperosmolarity. In all cases, only one cell was stained per slice to avoid misinterpretation in the field of diffusional coupling. Cells were uncoupled by adding halothane (diluted in absolute ethanol, final concentration 2 mM) in the bathing solution. Slices were continuously superfused with halothane 5 min before recording and remained under this condition until tissue fixation. After removal of the electrode from the recorded cell, neurobiotin was allowed to spread for 15 min into neighboring cells before final histological procedure. Briefly, tissues were fixed overnight in 4% paraformaldehyde at 4°C. After three 5 min washes in 0.1 M phosphate-buffered saline (PBS, pH 7.3), slices were treated for 30 min with 1% hydrogen peroxide and 10% methanol to block endogenous peroxidases. Slices were then washed three times in 0.1 M PBS, incubated overnight in avidin-biotin horseradish peroxidase (ABC kit, Vectastain Elite, Vector Labs) and 0.1% Triton X-100. Finally, slices were exposed to 0.05% diaminobenzidine tetrahydrochloride used as the chromophore, dried for several hours, mounted on slides and coverslipped in Eukitt for permanent storage and microscopic examination.

2.4. Statistical Analysis

Data were expressed as means \pm standard error of the mean and statistical significance was established using the Student's *t*-test or Mann-Whitney test in case of significantly different standard deviations.

3. RESULTS

3.1. Visual Selection of Cells

In preliminary experiments, the morphology of each cell visualized under DIC microscopy during patch-clamp recording was compared with the shape of the cell staining on-line with Lucifer yellow. Indeed, after achievement of the whole-cell configuration, Lucifer yellow was observed to fill almost instantaneously the recorded cell. Then, the dye fluorescence decreased rapidly within the target cell, this decline likely corresponding to a fast diffusion of the dye into neighboring cells (see below). In addition, because of fluorescence fading, morphological and coupling observations were found to be unstable within the 45 min duration of the experiment. Therefore, neurobiotin was routinely used for further analysis of recorded cells and study of intercellular communication. Comparison of DIC on-line microphotographs with neurobiotin staining after histological treatment and electrophysiological criteria of cell identification indicated that glial cells could be generally preselected for recording. This preselection was mainly based on the size of the cell. Thus, at all postnatal ages between PN8 and PN14, glial cell bodies were generally oval and significantly smaller than those of neurons (Fig. 1A, *see also* morphological data).

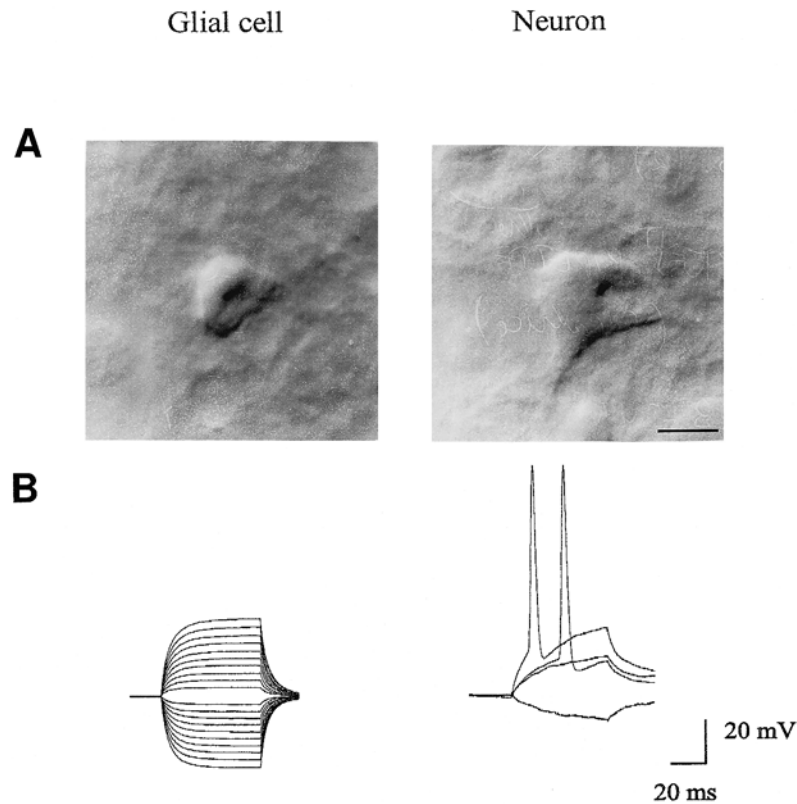


Fig. 1. Representative glial cells and neurons studied in striatal slices. **(A)** Differential interference contrast microphotographs showing that glial cells can be distinguished from neurons by their small cell body. Left panel: a glial cell. Right panel: a neuron. Scale bar, 10 μm . **(B)** Glial cells and neurons were also characterized by their electrophysiological properties examined in the whole-cell current-clamp mode. Left panel: responses of a glial cell to the injection of incremental current steps. No action potential was evoked by depolarizing steps. Right panel: traces from a neuron: a spike was emitted during depolarizing steps. Resting membrane potentials were -78 and -65 mV in the glial cell and the neuron, respectively.

3.2. Electrical Properties of Striatal Glial Cells: Current-Clamp Recordings

All patch-clamp recordings were performed within the striatum of animals aged from 8 to 14 d after birth. No difference was observed in the rate of success to establish the whole-cell configuration with different pipet solutions: standard solution alone or with the addition of Lucifer yellow or neurobiotin. Cell excitability was first examined in the current-clamp mode by analysis of voltage responses to the injection of several depolarizing and hyperpolarizing currents. (Fig. 1B). In general, the electrophysiological identification of the recorded cell, i.e., glia vs neuron, confirmed the selection achieved under DIC and infrared microscopy. Indeed, glial cells selected according to the small size of their soma never fired action potentials during the injection of large depolarizing currents (Fig. 1B, left panel), nor showed spontaneous potential changes in membrane potential (not shown). These data were obtained with

either standard pipet solution alone ($n = 11$) or pipet solutions containing a staining marker (Lucifer yellow: $n = 14$; neurobiotin: $n = 29$). No significant difference in the electrophysiological properties of the cells were observed with these different solutions. Therefore, all data obtained under current-clamp were pooled. Cells selected as neurons were recorded with pipets containing neurobiotin. As expected, these cells emitted typical action potentials in response to depolarization ($n = 8$; Fig. 1B, right panel). In addition, in 5 out of 8 cases, spontaneous spikes or spontaneous postsynaptic potentials were recorded at resting membrane potential (not shown).

When glial cells were recorded, in few cases (16% of the recorded cells, $n = 38$), the membrane potential shifted toward values more negative than -95 mV 15 min after the beginning of recordings. Thus, in all cells, the resting membrane potential (E_m) recorded during the first 2 min was only taken into account and found to be -82 ± 2 mV ($n = 38$). In addition, when recordings were performed with pipet containing neurobiotin, a shift of the resting membrane potential toward more positive values was observed (standard with or without Lucifer yellow: -87 ± 2 mV, $n = 13$; neurobiotin -79 ± 2 mV, $n = 25$, $p < 0.0001$).

When neurons were recorded, the resting membrane potential was more positive: -56 ± 5 mV ($n = 8$, $p < 0.0001$). Some neurons had a membrane potential of -50 mV or less and did not emit spikes in response to depolarizing stimulations even when prepolarized with sustained current injection. Consequently, these cells often associated with unstable whole-cell recordings were not taken into account. The neuronal input resistance did not significantly differ from that of glial cells when recordings were performed with either a standard pipet solution or a pipet solution containing Lucifer yellow (126 ± 31 M Ω , $n = 10$). In contrast, when cells were recorded with neurobiotin in the patch-pipet, the neuronal input resistance (151 ± 16 M Ω , $n = 8$) was higher than that of glial cells (89 ± 10 M Ω , $n = 24$, $p < 0.02$).

3.3. Voltage-Clamp Recordings

The voltage-dependent membrane properties of striatal glial cells ($n=38$) was investigated under voltage-clamp by stepping the membrane potential from -100 to $+90$ mV at a holding potential of -70 mV. Since similar observations were made with different pipet media, all data were pooled. Currents elicited in response to this family of voltage steps revealed different types of conductance profiles. "Passive" and "complex" cells were distinguished according to a previous classification originally proposed by Steinhäuser et al. (20,21). "Passive" profiles corresponded to a nearly ohmic and symmetrical behavior of the membrane currents in response to depolarizing and hyperpolarizing voltage steps ($n=24$). These "passive" cells were also characterized by a lack or a very slow decay time (several hundred milliseconds). In 16 out of 24 "passive" cells, pulse responses were followed by small currents at the end of the voltage step (Fig. 2A). In the eight remaining "passive" cells, currents recorded in response to voltage steps were associated with large, slowly decaying offset currents (Fig. 2B). In contrast, "complex" profiles consisted in voltage-activated currents superimposed on voltage-independent currents ($n = 14$, Fig. 2C). This observation was further confirmed in 14 out of 38 cells by a point-by-point analysis performed by adding positive and negative current traces (Fig. 2C, insert). In 12 out of 14 cases, "complex" cells were typically associated with small offset currents, which in few cases were found to be more pronounced in

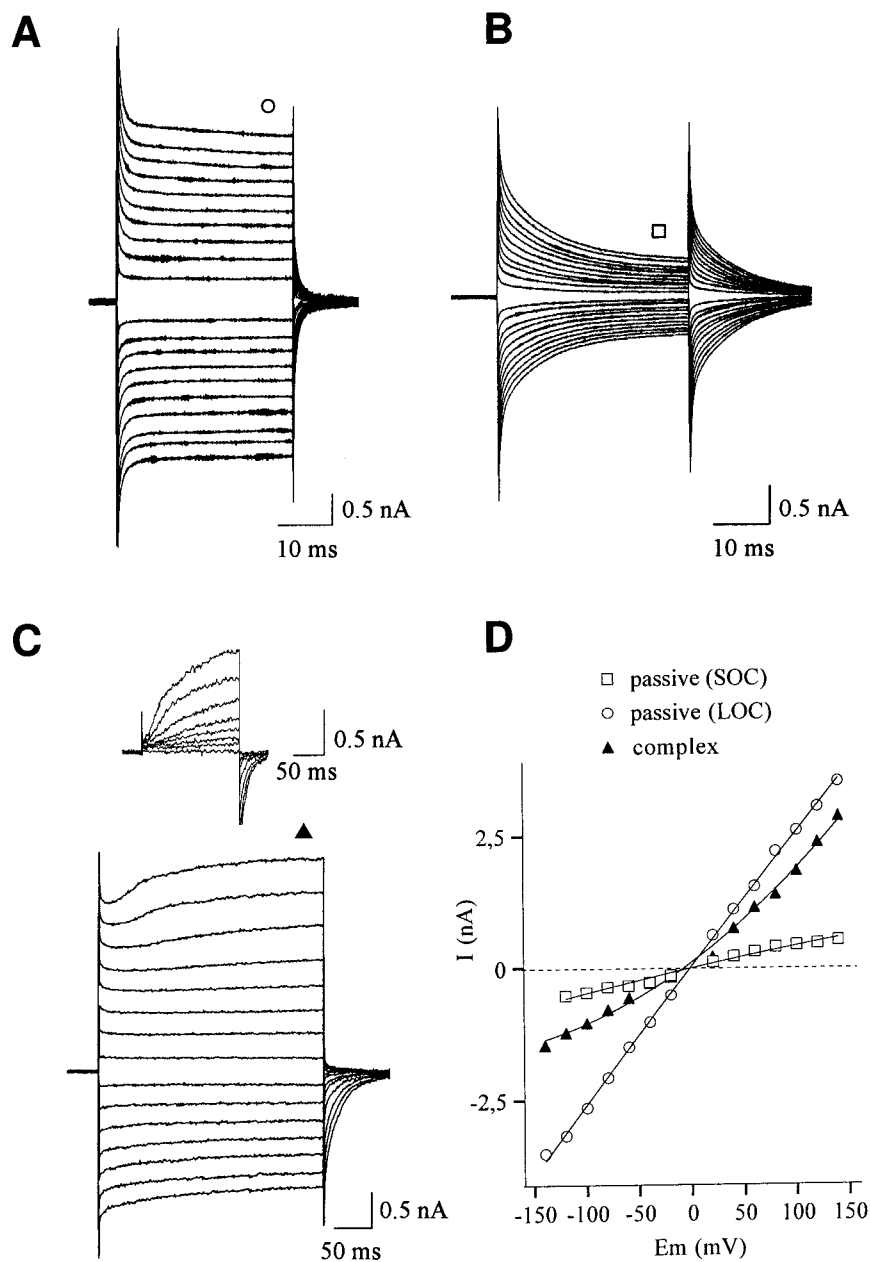


Fig. 2. Whole-cell current patterns of glial cells recorded at a holding potential of -70 mV, in response to de- and hyperpolarizing voltage steps ranging from -160 to $+40$ mV. **(A)** A “passive” cell with symmetrical currents showing neither voltage-, nor time-dependence. Small offset currents (SOC) are observed at the end of the stimulation pulses. **(B)** A “passive” cell expressing large offset currents (LOC) at the end of the stimulation. **(C)** A “complex” cell, endowed with voltage-dependent currents. Insert: the point-by-point addition of inward to outward currents reveals slowly activating outward currents of the delayed rectifier type. **(D)** I–V curves drawn from traces illustrated in **A** (white circles), **B** (white squares) and **C** (black triangles). Resting membrane potentials of these cells were -73 (**A**), -69 (**B**) and -77 mV (**C**).

Table 1
Different types of currents evoked in striatal glial cells by voltage steps between PN8 and PN14

	Cells with SOC		Cells with LOC	
	Symmetrical	Voltage-dependent	Small ratio	Large ratio
Time-independent	12	–	1	2
Slowly inactivating	10	–	3	2
Delayed rectifier (I_{DR})	–	2	–	–
Inward rectifier (I_{DR})	–	2	–	–
I_A	–	5	–	2
I_{Na^+} and I_A	–	3	–	–

Numbers represent the different types of voltage-gated and voltage-independent currents in response to voltage steps ranging from -100 to $+90$ mV from a holding potential of -70 mV. SOC, small offset currents; LOC, large offset currents. Voltage-dependent conductances were in most cases superimposed on symmetrical, ohmic conductances.

response to hyperpolarization (Fig. 2C). In 2 out of 14 neurons, “complex” cells were also characterized by large offset currents (*see* Table 1).

As previously reported, large offset currents are typically found in oligodendrocyte precursors and mature oligodendrocytes as identified by immunocytochemistry (22). Profiles of conductances in striatal glial cells were thus classified on the basis of their small (SOC) or large (LOC) offset currents. Decay time constants of offset currents were analyzed in current responses induced by a 50 mV step and calculated in the absence of deactivation of voltage-dependent currents. These decay times, often well fitted with a simple exponential function, were significantly larger in LOC than in SOC cells (9.2 ± 2.6 ms, $n = 7$, and 2.4 ± 0.8 ms, $n = 11$, respectively, $p < 0.05$). LOC cells were morphologically characterized by their parallel processes as revealed by microscopic analysis of neurobiotin injected cells. From these observations, LOC and SOC cells were considered to correspond to oligodendrocytes and astrocytes, respectively.

Further electrophysiological properties of “passive” and “complex” glial cells recorded from striatal slices were determined. Current-voltage (I/V) relationships established for responses measured at 50 ms before the end of the voltage pulse (250 ms duration) are illustrated in figure 2D for SOC and LOC “passive” cells and in figure 2C for “complex” cells. As expected from their ohmic properties, the I/V relationships were linear in SOC and LOC cells. In contrast, in “complex” cells, I/V curves were not linear. Indeed, in 9 out of 14 “complex” cells, outward currents were mainly observed whereas inward currents were also found in the five remaining cells. In two glial cells, outward currents were characterized by a delayed activation and no inactivation during 300–500 ms long depolarizing steps (not shown), reminiscent of that classically observed for delayed rectifying potassium currents. In 5 other glial cells, outward currents inactivated with a mean time constant of 17.2 ± 4.2 ms for depolarizing steps from -80 to -0 mV. The amplitude of these currents increased with conditioning hyperpolarizing steps more negative than -110 mV, which fits with the I_A type of potassium current. Furthermore, in 3 out of these 5 cells, these I_A -like outward current responses were superimposed with inward sodium currents. Finally, 2 other glial cells were

endowed with net inward currents inactivating at potentials more negative than -140 mV, reminiscent of an inward rectifying potassium conductance (see Table 1).

3.4. Morphological Analysis

Cell-filling with neurobiotin lasted from 3 to 30 min ($n=21$). The recording duration affected neither the staining intensity of somata and processes nor the tracer diffusion in the processes ($n=12$). Therefore, as initially observed *on line* with Lucifer yellow, neurobiotin appears to rapidly and completely fill the recorded cell after the membrane rupture.

Neurobiotin filling was considered to be adequate when no evidence of neurobiotin leakage into the extracellular space was found. Successful stainings were found in 18 glial cells and 3 neurons from a total of 29 control striatal cells recorded with neurobiotin. Average sizes of glial cell bodies were 6.5 ± 0.4 μm broad, 11.4 ± 0.8 μm long ($n=18$) whereas those of neurones were 10 ± 2 μm broad, 16.8 ± 1 μm long ($n=3$). Glial cell somata was elongated in most cases (6.2 ± 0.4 μm broad, 12.5 ± 0.8 μm long, $n=14$) and occasionally round (7.5 ± 1.1 μm diameter, $n=4$). Glial cells were also classified according to the morphological aspects of their processes. Several patterns were distinguished:

1. Branched processes orientated along one or two predominant directions (length range: 7 to 95 μm , $n=9$),
2. scarcely branched processes (maximal length range: 5 to 36 μm , $n=6$), and finally,
3. few short processes (length range: 1 to 3 μm , $n=3$).

In 7 out of 9 cells, either parallel elements elongating symmetrically from a thick bifurcating primary process were observed (total length range: 35 to 95 μm ; $n=3$) or a few shorter elements directly originating from the soma (maximal length range: from 11 to 55 μm ; $n=4$) were observed.

3.5. Intercellular Diffusion of Neurobiotin

Lucifer yellow was initially used for on-line estimation of gap junction-mediated coupling between striatal glial cells. Coupling was observed in 48% of the trials (23 experiments), the number of coupled cells ranging from 1 to 9. Halothane (2 mM), an inhibitor of gap junction channels in cultured striatal astrocytes (Mantz et al., 1993) was used to demonstrate that the staining of surrounding cells was mediated by gap junction channels. This uncoupling agent was applied 5 min before patch-clamp recording. As expected no dye diffusion was detected in the three experiments made in these uncoupling conditions.

Neurobiotin was then used instead of Lucifer yellow. When more than one cell was stained, the staining intensity was highly similar among stained cells, except at the cluster periphery where a weaker staining was detected (Fig. 3A). The recorded cell was identified according to its morphometric and electrophysiological characteristics (see above). When these identification criteria were not satisfied, the cell was eliminated from the coupling analysis. Cells were recorded in either the absence (control, $n=18$) or presence of halothane (2 mM, $n=15$), the mean recording duration being 16 ± 2 min (range from 3 to 30 min). In the absence of halothane, single stained cells were observed in 3 cases, and double or multiple staining in 15 other cases (Fig. 3A, left panel). In contrast, in the presence of halothane, 6 out of 15 cells were found to be

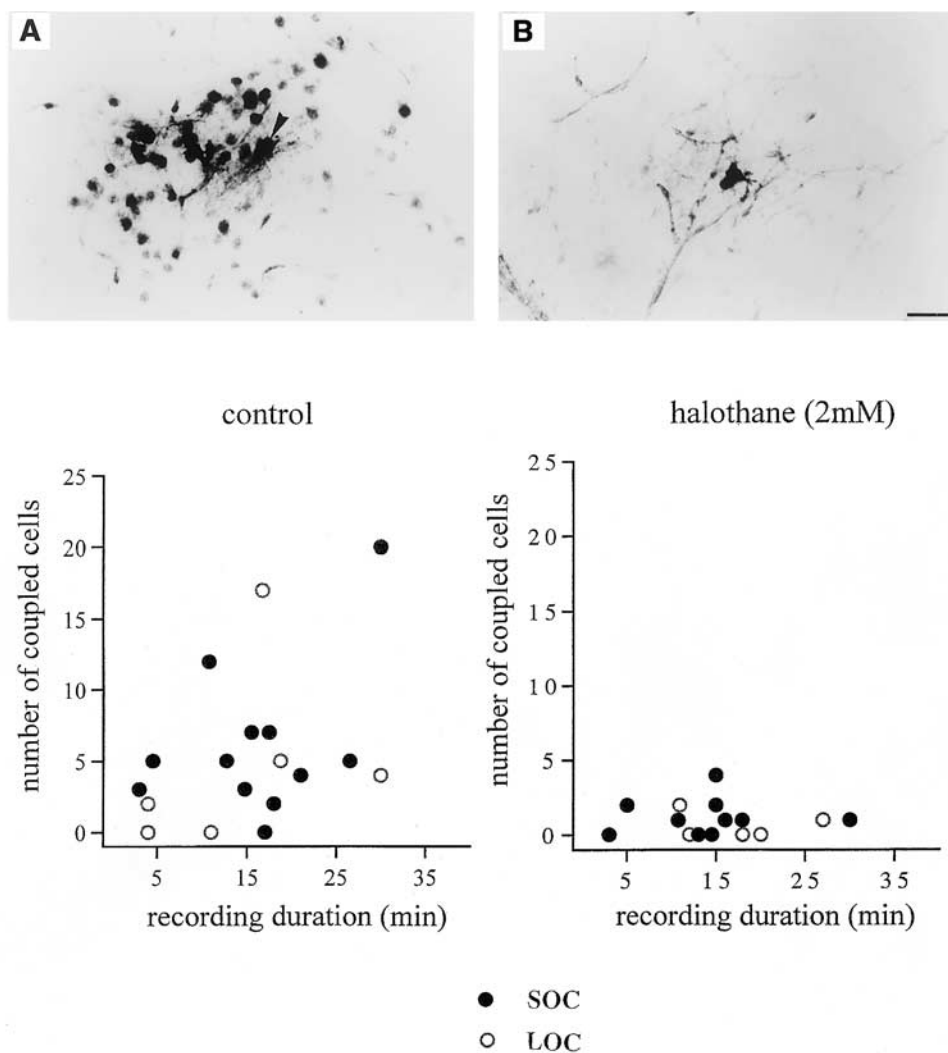


Fig. 3. Dye coupling of striatal glial cells and uncoupling effect of halothane. Upper part: microphotographs showing stained glial cells in the absence (A) or presence of halothane (B). Target cells (arrowhead) were recorded using a patch-pipet solution containing 0.5% neurobiotin in either the absence (control, left panel) or presence of 2 mM halothane (right panel). Lower part: quantitative representation of coupling under control conditions (left panel) and halothane perfusion (right panel). Number of coupled cells against recording duration is plotted for cells with small offset currents (SOC, black circles) and large offset currents (LOC, white circles).

single-stained (Fig. 3A, right panel) while the number of surrounding stained cells decreased from 7 ± 2 ($n = 14$) to 2 ($n = 9$) for control and halothane-treated cells, respectively ($p < 0.0005$, Mann-Whitney test).

Attempts were then made to look for a different sensitivity to halothane of the SOC and LOC glial cells. In SOC cells (classified as astrocytes), the number of associated stained cells decreased from 6 ± 2 ($n = 11$) to 1 ($n = 10$) ($p < 0.002$, Mann-Whitney test)

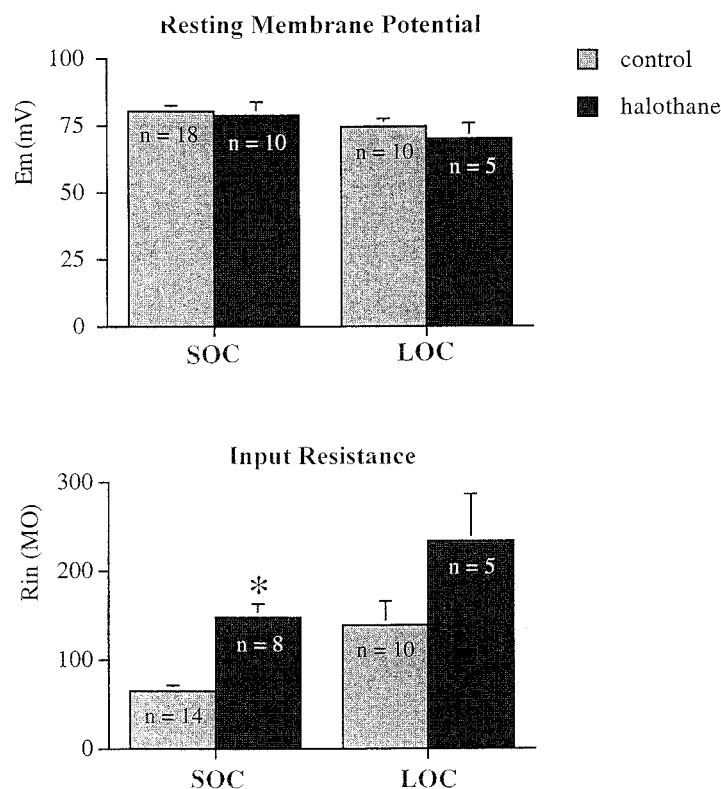


Fig. 4. Quantification of the effects of halothane on passive electrophysiological properties of gl SOC(astrocytes)and LOC(oligodendrocytes) glial cells.

the incidence of single staining being of 1 out of 11 and 3 out of 10 in control and halothane-treated slices, respectively. In LOC cells, the halothane uncoupling action was not significant, although 4 ± 2 ($n=7$) stained partner cells were observed in the absence of the uncoupling agent and 1 ($n=5$) stained neighbor cell under halothane (the incidence of single cell staining being 2 out of 7, and 3 out of 5 in control and halothane conditions, respectively). Altogether, these results indicate that multiple neurobiotin staining can be observed between striatal glial cells and that this intercellular diffusion is prevented, or at least reduced, in the presence of the blocker of gap junction channels.

3.6. Effects of Halothane on Passive Properties of Glial Cells

The effect of halothane on the passive membrane properties of glial cells were also tested in cells recorded with neurobiotin in the patch-pipette. In SOC cells (astrocytes), the membrane potential remained unchanged in the presence of halothane (-80 ± 2 mV, $n=18$ and -79 ± 5 mV, $n=10$, in control and halothane treated cells, respectively). Halothane treatment was also without significant effect on the resting membrane potential of LOC cells (oligodendrocytes) (-75 ± 3 mV, $n=10$, and -70 ± 6 mV $n=5$, in the absence and presence of halothane, respectively) (Fig. 4, upper panel). In contrast, halothane treatment increased the membrane input resistance in

astrocytes (SOC cells) from $65 \pm 7 \text{ M}\Omega$ ($n = 14$) to $148 \pm 15 \text{ M}\Omega$ ($n = 10$), in control and halothane treated cells, respectively but not in oligodendrocytes (LOC cells) ($139 \pm 27 \text{ M}\Omega$, $n = 8$, and $233 \pm 53 \text{ M}\Omega$, $n = 5$, in the absence and presence of halothane, respectively) (Fig. 4, middle panel). These observations agree with the greater uncoupling efficacy of halothane in astrocytes than in oligodendrocytes (see above). Finally, halothane decreased the amplitude of passive currents in astrocytes ($1.9 \pm 0.2 \text{ nA}$, $n = 18$, to $0.8 \pm 0.1 \text{ nA}$, $n = 11$ in absence and presence of halothane, respectively) but not in oligodendrocytes ($0.9 \pm 0.2 \text{ nA}$, $n = 8$, and $0.5 \pm 0.1 \text{ nA}$, $n = 6$, in absence and presence of halothane, respectively).

4. DISCUSSION

The present study demonstrates that in acute slices of the rat striatum, glial cells are connected by functional gap junction channels. This agrees with results of previous studies that indicated the presence of mRNAs coding for CX43, the astrocytic gap junction protein, and the expression of this protein within the striatum (9,10,24). Our conclusion based on results obtained both with Lucifer Yellow and neurobiotin thus differs from that of Rufer et al. (9), who failed to detect astrocytic dye-coupling in striatal slices using Lucifer yellow. This functional study completes our previous investigations which indicated that cultured striatal astrocytes are coupled by functional junctional channels mainly constituted of Cx43 (25). The present results obtained in the striatum provide further support for the existence of extensive glial communication through gap junctions, in several brain regions in vivo or in acute slices as already shown in the neocortex (26), the visual cortex (27), the hippocampus (6,5,8), and the cerebellum (7). Nevertheless, the extent of dye and tracer diffusion between striatal astrocytes appears rather moderate when compared with that observed in other structures. This was also found in our study in parallel experiments performed on cerebral cortical slices (data not shown). Such a difference agrees with results of comparative regional investigations on the distribution of Cx43 immunoreactive sites and mRNA (10,24), and with functional observations performed on cultured astrocytes from different brain structures (12,13).

One of our main concerns was to identify the recorded cells initially stained with the tracer. This identification was mainly based on electrophysiological and morphological criteria previously established in studies performed on the spinal cord, the red nucleus or the hippocampus (8,22,28,29). The absence of action potentials in response to injections of depolarizing currents allowed to conclude that recordings were made from glial cells instead of neurons. Oligodendrocytes were characterized by their long processes oriented in a parallel fashion to each other. Putative astrocytes were identified by their small and in most cases oval somata with short processes emerging in all directions. The morphological distinction between the two main types of glial cells was correlated in most cases with the presence of LOC and SOC, two electrophysiological properties previously proposed for the discrimination of mature oligodendrocytes and astrocytes, respectively (22). In addition, "passive" currents were mainly observed in oligodendrocytes while "complex" current/voltage relationships were found in astrocytes in agreement with previous published observations (8,22,28,29). Finally, the main conductances (I_A , I_{DR} , I_{IR} , I_{Na+}) known to be expressed in astrocytes and oligodendrocytes were also recorded from striatal glial cells.

Table 2
Morphological analysis of neurobiotin injected cells

	Size of somata (mean \pm s.e.m., μ m)		Size of processes (range, μ m)	
	Elongated, <i>n</i> = 14 (bride/length)	Oriented, <i>n</i> = 9	Diffuse, <i>n</i> = 6	Scarce, <i>n</i> = 3
Round, <i>n</i> = 4 (diameter)	6.2 \pm 0.4/12.5 \pm 0.8	7–95	5–36	1–3

In agreement with previous observations made on cultured glial cells, in striatal slices, astrocytes were more frequently and extensively coupled to neighboring cells than oligodendrocytes (30,31). The identity of the cells coupled with recorded glial cells remains to be established. Likely, this should be solved in the future by combining staining with the intercellular tracer and immunocytochemistry with either GFAP antibodies or oligodendrocytic specific makers. Presently, this identification was achieved on the basis of the size of the soma and the pattern of emerging processes of the coupled cells labeled by the tracer. According to these parameters and in agreement with previous observations made on cultured glial cells (31), the occurrence of homotypic (astrocyte-astrocyte or oligodendrocyte-oligodendrocyte) and heterotypic (astrocyte-oligodendrocyte) couplings could be expected. However, the existence of gap junction-mediated communication between a recorded glial cell with surrounding neurons cannot be excluded. Indeed, junctional coupling between astrocytes and neurons has been reported (32,33) and Cx43 immunoreactive staining between these two cell types has been detected *in situ* in the cerebral cortex (34).

Finally, the occurrence of functional gap junction channels between glial cells was also found to modify the basic electrophysiological properties of the recorded glial cells. Indeed, the input resistance was increased by about 50% and the amplitude of passive currents was reduced by 50% when junctional communication was inhibited by halothane. These observations demonstrate that electrical coupling through open gap junction channels may contribute to the passive electrophysiological properties of the astrocytes *in situ* (34). Accordingly, the presence of these intercellular communication processes should be taken into account when glial cells, and astrocytes, particularly, are recorded using the patch-clamp technique. This has been taken into account by D'Ambrosio et al. (8) who performed whole-cell recordings in hippocampal astrocytes by omitting ATP in their pipet solution, a procedure which depletes intracellular ATP and thus is expected to block gap junction channels (38).

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Glial Cell Involvement in Brain Repair and the Effects of Aging

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1. INTRODUCTION

The integrity of the central nervous system (CNS) is essential for the normal functioning of all aspects of animal behavior from the coordination of segmental muscle contractions in annelid locomotion to the development and maintenance of synaptic pathways involved in human memory and thought. The effects of brain injury are likely, therefore, to be devastating. It follows that repair of damage caused by disease or accident is of enormous importance to the well-being of the individual affected and yet the adult mammalian brain has poor powers of recovery from injury. If nerve tracts in the peripheral nervous system are damaged, it does not follow that the region of injury will not recover its full range of movement since axons in the periphery are able to regrow and reestablish innervation. A similar injury within the CNS, however, will not be repaired in a way that will lead to functional recovery despite the fact that CNS axons are intrinsically capable of growth and that sprouting and extension of neurites are initiated by injury. The main factor that controls the different outcomes in these two cases is the glial environment, which is permissive of axonal growth in the peripheral nervous system but checks growth within the CNS (1).

Glial cells were once regarded as mere supporting tissue for the neurones, a kind of “sponge” that held the neurones in place, provided nutrients, and mopped up waste products of neural activity. Thanks to the work of many researchers over the last three decades a much more realistic view of glial function has emerged and glial cells are now known to be crucially involved in the development and effective functioning of the nervous system throughout life. It follows, therefore, that perturbation of glial cell function is likely to have severe consequences: A view borne out by the links that have been established between glia and many neurological diseases including Parkinson’s disease, multiple sclerosis, progressive supranuclear palsy, and Alzheimer’s disease.

Although gross damage to CNS nerve tracts cannot be reversed, mechanisms exist for limiting and repairing damage caused by infection, oxidative stress, and mechanical lesions to the brain. Glial cells are intimately involved in these processes: They can proliferate in response to injury, act as controllers of inflammation, phagocytose damaged tissue, secrete neuroprotective and neurotrophic molecules, form boundaries to isolate regions of damage and help to maintain ionic homeostasis within damaged

regions of the brain. However, they can also promote dangerous levels of inflammation, secrete neurotoxic molecules, inhibit neurite growth, and promote demyelination. In this chapter we will describe injury-induced cellular responses associated with glial activation and consider both the balance that exists between beneficial and harmful effects of activation and the effects of aging in weighting this balance.

2. BRAIN INJURY AND ITS IMMEDIATE EFFECTS

The main experimental technique used to induce *in vivo* CNS injury in vertebrates is occlusion of cerebral arteries for shorter or longer periods thus causing different levels of ischemia throughout the brain and mimicking the effects of stroke injury. Some studies have also been carried out using mechanical means, e.g., stab-wounds to the brain and the severing of specific nerve tracts, or by chemical lesioning, e.g., injection of kainic acid or 3-nitropropionic acid into the brain. Selective lesioning of glial elements has also been carried out in invertebrates where the layout of the nervous system enables localized destruction of glia through the application of ethidium bromide to regions lacking nerve cell nuclei. In this chapter, we will largely consider the sequence of repair that follows ischemia but will include data from studies of other types of brain injury where these shed light on additional repair processes.

The immediate effect of restricting the flow of blood in the carotid arteries is to reduce oxygen levels throughout the brain. The resulting anoxia causes a rapid decline in normal energy supplies and a switch to anaerobic metabolism in which lactate is produced as a by-product. These events lead to failure of the Na^+K^+ -ATPase exchanger in neuronal membranes and an accumulation of axoplasmic Na^+ via voltage-gated Na^+ channels. The combination of high internal concentrations of Na^+ and a concomitant depletion of K^+ depolarizes the neuronal membrane. This stimulates a reverse Na^+ - Ca^{2+} exchange, and the resulting sharp rise in Ca^{2+} levels within the neurone activates Ca^{2+} -dependent enzymes such as phospholipases, calpain, and protein kinase C that can cause irreversible damage and death of the affected neurones (2,3). Not all regions of the brain are equally susceptible to ischemia: CA1 hippocampal neurones in adult rat brains are killed by a 10 min exposure to ischemia but there is little or no damage to neurones in other regions of the brain (4). Similarly, in organotypic cultures, CA1 hippocampal neurones are damaged by short (7 min) exposure to ischemia while CA3 neurones and the most susceptible neocortical neurones withstand longer periods (up to 30 min) of anoxia. Dentate gyrus and some neocortical neurones are even more resistant and are only damaged after 60 min exposure to anoxic conditions (5).

Glial cells are considered to be more robust than neurones in their response to ischemia but, following even brief periods of global ischemia, some damaged glial cells appear in the cortex and thalamus. Most of these are oligodendrocytes, possibly because they are susceptible to glutamate released from damaged neurones. In the case of prolonged ischemia or mechanical injury, external levels of glutamate may be augmented by release from astrocytes at the site of injury given that a gradual loss of their transmembrane Na^+ -gradient would lead to conditions favoring carrier reversal and the extrusion of glutamate from the cells (6). Since glutamate is also a ligand of the *N*-methyl-D-aspartate receptor on neurones, these elevated levels of glutamate would have the effect of inducing further excito-toxic neuronal injury and death, thus extending the effect of the initial lesion (7)

Mechanical injuries such as stab wounds destroy neural tissues directly and introduce breaches in the blood-brain-barrier that threaten the homeostasis of the brain. Death of neurones and glial cells is augmented as blood leaks into the damaged region changing the ionic environment around undamaged cells and inducing fluctuations in membrane potential and a cycle of events resembling that outlined above. Uncontrolled entry of circulating lymphocytes and other cells of the immune system to previously protected neural tissue may mount an autoimmune response in addition to the inflammatory reaction set in motion by the resident immune system of the brain.

Chemical lesions are designed to cause specific types of injury e.g., the use of serotonin neurotoxin to cause death of serotonergic neurones without gross damage to other cells or kainic acid injection to cause selective damage to hippocampal CA3 pyramidal cells. Similarly, axotomy of specific nerves, such as the facial nerve has been used to produce limited damage in the region of the brain to which the damaged axons project. Localized application of ethidium bromide to interganglionic connectives within the insect CNS enables selective destruction of glial cells and allows glial response and repair to be studied in the absence of neuronal damage. All types of injury appear to initiate a similar chain of glial response.

3. GLIAL RESPONSES TO INJURY

3.1. *Glial Types*

In discussing glial responses to damage it is necessary to differentiate between the three major types of glia recognized in the brain:

1. Microglial cells form between 5 and 20% of the neuroglial population and differ from other glial cell types in the brain in being derived from mesenchymal tissues. The relationship between microglia and circulating macrophages has been discussed by Perry and Gordon (8) and it is clear that microglial cells form the main element in the immune system of the brain. They are particularly responsive to small changes in their environment, carry macrophage markers on their cell surface and respond rapidly to damage. They are also able to change their morphology, act as phagocytes and secrete a range of cytokines and growth factors that modulate neural repair (9).
2. Astrocytes in the intact brain are largely responsible for maintaining its homeostasis both by forming the blood-brain-barrier and by uptake and secretion of molecules in the extracellular fluid e.g., astrocytes metabolize glutamate released at nerve endings before it can reach cytotoxic levels and they contain glycogen stores that can be released to supply neuronal energy demands. In addition, astrocytes have been shown to release neurotrophic factors that support neuronal survival and sprouting, which may be of major importance during both development and neural repair.
3. Oligodendrocytes are the glial cells most intimately associated with neurones within the brain, they are responsible for myelination, provide metabolic support for neurones and are closely involved in signaling between neurones and glia via voltage- and ligand-gated ion channels (10,11) They do not appear to play a role in the glial reactivity that follows brain injury, but are a factor in preventing repair in the CNS since myelin-associated proteins can inhibit neurite growth. As described above, they are the glial cell type most susceptible to injury by anoxia but such damage can be repaired by recruitment from a limited pool of progenitor cells resident within the brain (12)

3.2. *Pattern of Glial Cell Response to Injury*

The general response of glial cells to neural tissue damage is similar over a range of different injuries although the level of reactivity depends on the severity of the injury. There is an initial activation of resting microglial cells, which involves a change in cell shape and accumulation of reactive microglia in and around the site of injury. This is followed by a period of astrogliosis during which increasing numbers of activated astrocytes appear within the brain. The majority of these cells are associated with the damaged tissues, and their most obvious activity is the formation of a glial scar. The recruitment of cells in these two processes is a prerequisite for the repair processes that are set in train by cell damage and death within the brain. The initial inflammatory reaction in which they are involved is an important event since the level of this response may determine the success of any future repair. A combination of upregulation of signaling proteins, cytokines, neurotrophic proteins, and other proteins involved in the cycle of events eliciting brain repair is accompanied by apoptotic cell death and the induction of phagocytotic activity to clear debris within the lesion. Tight control of these processes is essential to prevent the inflammatory process itself causing further and more widespread cellular damage. Consequently, a considerable amount of research has been directed toward understanding the processes of microglial and astrocytic activation, the results of which we summarize here.

3.3. *Activation of Microglial Cells*

Activation can occur extremely rapidly and is often detected before damage to neurones is visible; Ivacko et al., (13) using Griffonia simplicifolia B4-isolectin to identify microglial cells were able to determine subtle changes in their appearance within 10 min of administering hypoxic injury to young rats. Four hours after injury, activated microglial cells could be identified in the lesion zone and the number of microglial cells present continued to rise with numbers peaking 2 to 4 d after injury. In a similar study of brief ischaemic injury in rat brain, proliferation of microglial cells was reported to peak seven days after injury (14). Intraventricular kainic acid injection also results in the accumulation of microglial cells around damaged pyramidal neurones within 3 h and, prior to this, alterations in the shape of the microglial cells to an amoeboid form are detectable (15). The fact that microglial cells play a continuing role in brain repair processes is demonstrated by a study in which kainic acid lesion-induced changes in the morphology and number of microglial cells in the mouse brain were monitored over a period ranging from 12 h to 81 d postlesion (16). This showed a decrease in the number of cell processes and an accompanying rounding of the microglial cell 12 h post injury. Short, thick processes unlike those of resting microglia began to re-extend from the cell 48 h postinjury and very gradually changed to give a return to the normal, resting morphology after approximately one month. Over the same period, changes in the number of microglial cells in regions of the brain associated with damaged neurones were apparent. Cell counts around the lesion remained stable for two days and then increased from approx 100 cells/mm² to 500 cells/mm² 3 d postlesion. The number of microglial cells present continued to rise for a further two days and remained high for approximately a month before gradually falling to their original level.

Observations such as these raise the question of whether this increase in reactive microglial cell numbers is the result of migration to the site of injury from undamaged regions of the brain, of proliferation of resident microglia or of the recruitment of monocytes across the blood-brain-barrier and their transformation into microglial-like cells. It seems likely that in the case of noninvasive brain injury the initial accumulation of cells in and around the lesion is due to microglial cell migration since Akiyama et al. (15) noted a reduction in the number of microglial cells in adjacent regions concomitant with the appearance of cells in the lesion zone. In addition, the ability of activated microglial cells to migrate toward injured neurones has been demonstrated *in vitro* in hippocampal slice cultures (17). Proliferation of microglial cells has also been identified at sites of injury in rat brain (18,19). Following cortical stab wounds maximal microglial proliferation is reported to occur between 2 and 3 d postlesion (20), coinciding with the timing of the increase in cell numbers described by Andersson et al. (16). Although such studies indicate that cell division contributes to the long-term increase in microglial cell numbers, they do not rule out the possibility that blood monocytes may also be involved in the secondary increase in microglial cell numbers seen between 2 and 5 d postlesion. Even in the case of treatments that do not compromise the blood-brain-barrier, monocytes are recruited into the brain within 2 d of injury and subsequently transform to macrophages (21). More recently Akiyama et al. (15) have shown that vascular endothelial cells begin to express ICAM-1 approximately six h after ischemic injury with levels gradually increasing over the next 42 h. As a ligand for leucocyte function associated antigen (LFA)-1, the expression of ICAM-1 precedes the accumulation of leucocytes in the brain vasculature. These then pass through the blood-brain-barrier to infiltrate the brain, where they may assume some of the functions of reactive microglia. A similar involvement of blood cells in repair of selective glial lesions to cockroach CNS was demonstrated by Treherne et al., (22) who showed that hemocytes pass through the intact neural lamella, accumulating in the site of injury where they transform into granule-containing cells that play a crucial role in subsequent repair processes.

3.3.1. Upregulation of Microglial Proteins

The transformation from resting to reactive state is accompanied by upregulation of a number of proteins, some of which are specific to microglia, although others are also expressed by reactive astrocytes (Table 1). In keeping with their immuno-protective role, many of the proteins on reactive microglial cells are associated with aspects of the immune reaction. Thus, macrophage scavenger receptors appear on microglial cells within 24 h of kainic acid injection into rat brain, consistent with the view that microglial cells take on a phagocytic role and assist in the removal of debris from damaged cells (23). The class II major histocompatibility complex (MHC class II), which is expressed at low levels on at least some resting microglia, increases in response to infection or injury. The degree of upregulation is controlled so that although, for example, a viral infection may induce almost all microglia to become MHC class II positive, limited brain damage following peripheral axotomy results in upregulation confined to the region of the brain into which the damaged axons project. The role of MHC class II in the peripheral immune system is to present antigen to helper T-lymphocytes, but it is a matter of current debate whether it has this function in the CNS. In inflammatory

Table 1
Glial factors upregulated in response to brain lesion

	Factor	Function	
Activated Microglia cytokines:	IL-1	– enhances dopaminergic neuron sprouting	
	IL-1 α	– activates astrocytes	
	IL-1 β	– upregulates β -amyloid precursor and S110 β proteins – proinflammatory – inhibits Ca ²⁺ influx, protein kinase activity, ACh and glutamate release from neurons – induces reactive oxygen species formation	
ICE:	IL-6	– appears prior to apoptosis; possible mediator of inflammation – increases blood brain barrier permeability – proinflammatory – role in migration and differentiation of oligodendrocyte precursors	
	IGF-I, II	– neuroprotective – neurotrophic	
	TGF β -1	– immunosuppressive – inhibits ICAM-1 expression – chemotactic for astrocytes and microglia – inhibits astrocyte proliferation – induces differentiation of oligodendrocyte precursors – upregulates GFAP expression	
	β -chemokines		– neuroprotective
			– monocyte chemoattractant – macrophage inflammatory protein

Table 1
(Continued)

	Factor	Function
cell adhesion molecules: macrophage-associated antigen:	ICAM-1	– upregulated by LPS
	MHC I	– important for lymphocyte activity in CNS
	MHC II	– antigen-presenting molecule
	complement type-e receptor	– antigen presentation
	leucocyte common antigen	
extracellular matrix molecules:	macrophage scavenger receptor	
	laminin	– enhances neurite outgrowth
	fibronectin	– promotes oligodendrocyte migration
others:	osteopontin	– glial scar formation
	cytosolic phospholipase A	– breakdown of phospholipids
	HSp 70	– neuroprotective
	thromboxane	
	ATP-hydrolysing enzyme	– neuroprotective via release of adenosine
	amyloid precursor protein	
	cystatin C	– cysteine protease inhibitor
	prostanoids	– neuroprotective
	NO synthase	– upregulates nitric oxide, a cytotoxic signalling molecule
	CCAAT-enhancer	– sequence-specific binding protein, possible regulator of gene expression
Activated Astrocytes cytokines:	binding protein α	
	bFGF	– trophic for neurons, glia and endothelial cells – induces activated morphology in astrocytes – supports oligodendrocyte growth – protective against reactive oxygen species
	FGFR	
	EGFR	

(continues)

Table 1
(Continued)

	Factor	Function
Activated Astrocytes		
	BDNF	– supports axonal regrowth
	NGF	– enhances oligodendrocyte survival
	CNTF	– oligodendrocyte differentiation, astrocyte activation
	NT-3	– neurotrophic
	IGF-1	– neuroprotective
		– neurotrophic
	IGF-II	
chemokines:	MCP-1	– recruitment of blood-derived inflammatory cells
	IP-10	– recruitment of blood-derived inflammatory cells
cell adhesion molecules:	VCAM-1	– aids leukocyte entry through blood brain barrier
	ICAM-1	
intermediate filament proteins:	GFAP	– morphological changes
	nestin	– morphological changes
	vimentin	– morphological changes
extracellular matrix molecules:	vitronectin	– supports astrocyte migration
	laminin	
	fibronectin	– promotes oligodendrocyte migration
	tenascin-C	– inhibits oligodendrocyte migration
antioxidants:	(mSOD	
	glutathione peroxidase	– elimination of free radicals
	catalase	– protection against oxidative stress
	glutathione	– neuroprotective
	hemeoxygenase 1	
	NO synthase	– upregulates NO production
	NO synthase)	– signalling molecule
		– cytotoxic

cytosolic phospholipase A	– phospholipid breakdown
substance P	– regulator of inflammation, wound healing and immune response
HS _p 70	– neuroprotective
calbindin D28K	– neuroprotective
	– Ca ²⁺ homeostasis
L type Ca ²⁺ channels	– Ca ²⁺ homeostasis
	– Ca ²⁺ signalling
endothelin β receptor	– mitogenic to astrocytes
	– mediates actin reorganization
NF _k B	– gene transcription factor

Abbreviations:

ACh	acetylcholine	IL	interleukin
BDNF	brain-derived neurotrophic factor	IP-10	IFN-γ-inducible protein
CNTF	ciliary neurotrophic factor	LPS	lipopolysaccharide
ICAM-1	intracellular cell adhesion molecule 1	MCP-1	monocyte chemoattractant peptide-1 GFAP
ECM	extracellular matrix	NGF	nerve growth factor
EGFR	epidermal growth factor receptor	NO	nitric oxide
bFGF	basic fibroblast growth factor receptor	NT-3	neurotrophin 3
GFAP	glial fibrillary acidic protein	MHC	major histocompatibility complex
HS _p 70	heat shock protein	SOD	superoxide dismutase
ICE	interleukin-1 β converting enzyme	NFκB	nuclear factor kappa B
IGF	insulin-like growth factor		

brain lesions, the apoptosis of T cells that occurs in neuroectodermal parenchyma does not depend on antigen presentation by glial cells and the T cells that infiltrate the brain are destroyed whatever their antigen specificity or level of activation (24).

A number of macrophage cytokines are expressed by microglia and the timecourse over which they are upregulated *in vivo* is consistent with the view that they are involved at early stages of glial activation. Transforming growth factor- β 1 (TGF- β 1) mRNA is expressed by activated microglial cells within 6 h of ischaemia and since TGF- β 1 has an inhibitory effect on astrocyte proliferation (25), it may have a role in limiting potentially damaging inflammatory responses after injury. The continued expression of TGF- β 1 by both microglial cells and macrophages for periods of up to 3 mo suggests that it also has a role in long-term repair and remodeling of damaged neurones. Cytokines also have mitogenic, neuroprotective, and neurotoxic properties when tested on cultured cells but it is difficult to predict how the effects of different cytokines will be modulated within the complex environment of the brain. There are increasing numbers of *in vivo* studies, however, to support the view that cytokines play an important role in CNS repair. For example, the finding that injection of interleukin-6 (IL-6) into rat brain reduces the level of neuronal damage produced by prolonged ischemia coupled with the fact that increased levels of endogenous IL-6 activity are measurable in ischemic regions of the brain within 2 h of damage (26), suggest that upregulation of IL-6 in activated microglia has a physiological role *in vivo*. Similarly, macrophage colony stimulating factor (M-CSF) has been shown to have a mitogenic and morphological effect on microglial cells *in vitro*, although *in vivo* infusion of a related colony stimulating factor, GM-CSF, results in an increase in microglial cells in the treated area (27). The finding that a mouse strain carrying a mutation silencing the gene encoding M-CSF fails to show the usual reactive microglial proliferation following facial nerve axotomy (28) also indicates that colony stimulating factors are important for microglial responsiveness.

Activated microglia *in vivo* can secrete growth factors including insulin-like growth factor (IGF-1), epidermal growth factor (EGF) and nerve growth factor, and it is likely that these have a neurotrophic role. A recent paper shows that 3 d after lesion mRNA for IGF-1 is present in microglial cells in regions of delayed neuronal cell death. A concomitant increase in IGF-binding protein in reactive astrocytes closely associated with surviving neurones suggests that microglial IGF-1 is transferred to these astrocytes and may be used by them for the continued support of the neurones (29).

Other proteins expressed by activated microglial cells may be involved in limiting neuronal damage by other means. An example of this is the proposed role of microglial cells in the generation of adenosine from ATP. Adenosine has neuroprotective properties and increased levels of adenosine are found in damaged areas of ischaemic brains. Although these may partly result from release by damaged cells, it has also been shown that upregulation of ectoapyrase and ecto-5'-nucleotidase, which together can hydrolyze nucleoside 5'-mono-, di- and triphosphates, occurs within 2 d of ischaemia. They are still present 28 d post-lesion, particularly in the vulnerable CA1 hippocampal region, and their pattern of expression coincides with the distribution of markers for reactive glia, especially those associated with microglia (30). Extracellular matrix (ECM) molecules such as laminin and fibronectin are expressed by microglial cells

after CNS injury and are known to influence neuronal growth *in vitro*. Their potential role *in vivo* is discussed in relation to astrocyte activation in a subsequent section. Another ECM molecule, osteopontin, provides an example of the inter-relationship between microglial and astroglial cells following injury. Osteopontin mRNA is expressed *de novo* by microglial cells at the margin of the damaged region within 3 h of ischaemic lesion. The protein itself subsequently occurs in this same area within 24 h and is later also expressed within the lesion zone. The integrin receptor for osteopontin, $\alpha(v)\beta(3)$, is expressed by astrocytes 5–15 d post-lesion, initially at a distance from the osteopontin-expressing microglia but by day 15 coincident with them (31). This observation suggests that activated microglia may initially be responsible for delineating the area of the lesion by laying down extracellular material with which migratory reactive astrocytes will then interact during glial scar formation.

3.4. Activation of Astrocytes

Reactive astrocytes begin to appear in the brain shortly after the initial activation of microglial cells (32). Morphologically these astrocytes are characterized by an overall increase in size which includes lengthening and thickening of cellular processes. In addition, glial fibrillary acid protein (GFAP) expression is upregulated, the number and size of the mitochondria present increases and the endoplasmic reticulum becomes enlarged. When rats are subjected to brief ischaemic injury, reactive astrocytes appear at the site of injury within a week of damage, with numbers peaking at around 2 wk (33), which is in the same range as the glial repair processes that follow glial lesioning in insect CNS. An increase in perineurial glial cells is apparent in the cockroach within four days of chemical lesion with numbers peaking after 6 d, at which time there is a further recruitment of subperineurial glia in the lesion zone, thus extending the period of gliosis to approximately 2 wk (34). Much more rapid induction of astrocytic gliosis occurred in hippocampal and neocortical organotypic cultures subjected to histotoxic ischaemia where gliosis could be detected within 30 min of injury (5).

The extent of astrogliosis within the brain varies with the type and severity of injury and tends to be more widespread than the distribution of activated microglia. In general, astrogliosis following ischaemic injury is largely restricted to the area of the lesion but, following a stab wound to the cerebral cortex, astrogliosis may occur over a widespread area with GFAP-positive astrocytes even present in the hemisphere contralateral to the wound (35). Such distant responses may depend on the extensive communication system, based on changes in intracellular free Ca^{2+} concentrations transmitted from cell to cell through gap junctions, that exists in astrocytes (36). Gap junctions can carry apoptotic signals from damaged cells to adjacent healthy cells causing widespread death at sites distant from the original lesion (33), but the importance of such signaling in extending and controlling cell damage within the CNS is not known. During the early stages of neuronal degeneration following ischemic injury neurones can trigger Ca^{2+} waves in adjacent astrocytes via gap junctions or glutamate release (37) and although these signals may initiate cell death it is also possible that they trigger astrogliosis. In this context, it may be relevant to note that, a calcium-binding protein, calbindin-D28K, normally present in neurones is also found in reactive astrocytes in the CA1 subfield after ischaemia (38).

Whatever the signal pathways involved, the appearance of large numbers of activated astrocytes within a lesion must involve transformation of a preexisting population of astrocytes and/or glial cell proliferation. As with microglia, it appears that both processes occur but the balance between them varies in different situations. Eclancher et al., (39) reported that some 22% of GFAP-positive astrocytes incorporated ³H-thymidine following an electrolytic lesion of rat brain. Similar evidence has been obtained for astrocyte division following ischemia, mechanical wounding and chemical lesioning. In other studies, however, attempts to label astrocytes with ³H-thymidine have shown little or no incorporation (40,41).

3.4.1. Upregulation of Astrocyte Proteins

In addition to upregulation of GFAP, which is the most commonly used marker of astrocyte activation, a number of other proteins are upregulated or newly expressed (Table 1). The functions of these proteins fall into four broad areas:

1. Containment of the site of injury,
2. Regulation of inflammation,
3. Promotion of neuronal survival,
4. Reduction of oxidative stress.

Morphological changes, cell migration to lesion sites, cell proliferation, and production of extracellular matrix molecules are all likely to be involved in restricting the site of lesion by forming a glial scar. The expression of both GFAP and another intermediate filament protein, nestin, is linked with the change in shape that accompanies activation. This process must involve reorganization of other cytoskeletal elements by changes in the disposition of actin and actin-binding proteins, for example, and the effects of this on surface membrane protein activity have yet to be investigated. A recent *in vitro* study has, however, demonstrated a role for the small GTP-binding protein Rho in modulating changes in astrocyte shape (42). Rho is involved in the regulation of F-actin in some cell types, where its activity is modulated by components of the extracellular matrix. The production of ECM molecules may thus have an effect on both the morphology and the migration of astrocytes since, in addition to the Rho pathway, ECM molecules that bind to integrins on the cell surface influence cell shape in some systems, while vitronectin has been shown to support migration of astrocytes *in vitro* in the presence of TGFβ1. Secretion of ECM by activated astrocytes will also influence other aspects of the repair process: Different ECM proteins can exert modulatory effects on neuronal and oligodendrocyte cell extension and affect the migration of oligodendrocyte precursor cells. Laminin, which promotes neurite outgrowth in developing brain, is transiently expressed by activated astrocytes (43) and there may be a causal relationship between the secretion of laminin by astrocytes and the short-lived neurite sprouting seen after CNS damage. It may be significant that in species such as frog and goldfish where the adult CNS is permissive of axonal growth laminin is expressed in the brain throughout life (44). Laminin, together with fibronectin also supports the migration of oligodendrocyte precursor cells, however, a third ECM molecule, tenascin-C, inhibits this migration (45) and its presence in the glial scar may prevent remyelination of axons within the lesion.

The effects of the extracellular matrix are modulated by cytokines, a number of which have been implicated in the processes accompanying astrocyte activation. For example, bFGF enhances the ability of astrocyte-secreted ECM to support oligodendrocyte outgrowth *in vitro* (46), whereas TNF α blocks astrocyte migration, even in the presence of a migration-inducing cytokine, TGF β , unless vitronectin is also present (47). Cytokines are involved in many other aspects of the response of the brain to lesion including control of inflammation, effects on proliferation and migration of all glial cell classes and production of nitric oxide and reactive oxygen species (48). The diversity of cytokine effects is demonstrated by the fact that a single cytokine, bFGF, is reported to protect neurones from glutamate toxicity, upregulate antioxidant defenses via glutathione, induce an activated morphology in astrocytes and have a potent trophic effect on neurones, glia, and endothelial cells.

A variety of other astrocyte-associated proteins affect these same processes: Substance P modulates inflammation, heat shock proteins protect neuronal function, and heme oxygenase-1 protects against oxidative stress, whereas the upregulation of L-type Ca⁺ channels may regulate homeostasis and enhance release of neurotrophic agents as well as being involved in signaling in astrocytic networks.

As the above description of microglial and astrocyte activation indicates, glial cells mount a robust response to injury and are able to control and reverse at least some of the effects of brain injury. The level of permanent damage inflicted by a brain lesion depends upon the number of neurones that are killed; astrocytes and microglia are capable of proliferation, oligodendrocytes can be replaced by differentiating precursor cells but neurones cannot be replaced. In addition, the more widespread the damage to neurones and myelin-producing cells, the more intense the inflammatory response is likely to be, culminating in the destruction of previously undamaged neurones as activated microglial cells attack their myelin sheaths. The major role of glia may, therefore, be to control inflammation by rapidly isolating the region of damage from the rest of the brain thus restricting cytokine activation to a limited area and enabling a balanced process of tissue destruction and repair to proceed. The effects of aging on the maintenance of this balance are likely to be variable and complex.

4. CHANGES IN THE AGING BRAIN THAT MAY AFFECT REPAIR PROCESSES

Studies of the ways in which the CNS changes during normal aging are less common than studies of the effects of diseases of the brain and there have been few direct studies of the effects of age on responses to lesions. However, by evaluating results from studies that have clarified aspects of the aging processes in nondiseased brains, it is possible to select age-related changes that are likely to have an impact on glial responses to brain injury. Although there is some work on aging in the CNS of normal human subjects most studies in this area use rodents as models. Work with rats and mice of different ages, as well as studies on senescence-accelerated mice, have provided much information on the effects of age on brain morphology and function, whereas *in vitro* studies have enabled examination of the effects of cell "age" on cell-cell interactions. It is worth noting that rodents are often maintained to advanced ages by strictly controlling their food intake, but since recent work has shown that dietary restriction influences

various aspects of brain aging through reduction of oxidative stress (49), such animals are not good models of normal aging processes.

4.1. Morphological Changes in Aging Brain

Overall changes in brain volume as well as more specific changes in the number and morphology of brain cells have been reported from studies of aging subjects but the extent of such changes is unclear. There is a general consensus that as the brain ages there is a gradual decrease in neuronal cell number, especially among cholinergic neurones, probably accompanied by a reduction in dendritic spine density and an increase in glial cell numbers. Changes in oligodendrocytes have not been widely reported although some vacuolation and demyelination may take place in older brains. With the development of new technologies including improved methods for estimating cell numbers and the availability of specific glial markers, more accurate studies of neural cells have become possible, and it is instructive to look at some of the most recent examinations of changes in cell types with aging.

A study in rats revealed no differences in cortical volume or neuronal density between young (4–6-mo-old) and aged (30–32-mo-old) rats but a decrease in the area occupied by neuronal somata was identified in layers II–VI. This was accompanied by changes in neuronal shape in these same layers and a decrease in neuronal nuclear area in layer VI. Glial density increased by ~17% throughout the cortical layers (50). This increase was subsequently shown to result from changes in the number of astrocytes present, appearing as astrocytic clusters in older brains, whereas the numbers of microglia and oligodendrocytes remained constant between the two groups. However, changes in the morphology of microglial cells were noted, the significantly larger numbers of cytoplasmic inclusions present in older animals presumably reflecting increased phagocytotic activity with age (51). A similar result was obtained by Amenta (52), who examined the number and morphology of GFAP-positive astrocytes in the frontal cortex and CA1 hippocampal subfield of adult (12-mo-old) and aged (24-mo-old) rats. An age-related increase in the number and size of GFAP-positive astrocytes was found in the two regions but they differed in that, although increases in cell numbers was more apparent in the CA1 subfield, increased astrocytic size was more noticeable in the frontal cortex. Although comparable results have been found in a number of studies, they are by no means a universal finding. An examination of the supraoptic nucleus region in groups of rats ranging in age from 5 to 24 mo failed to find any significant changes in the number of neurons or astrocytes between groups, although some degenerating neurons were observed in older animals. This same study does, however, describe age-related changes in the morphology of astrocytes including an increase in nuclear size associated with the appearance of nuclear bodies, clear patches within the nucleoplasm and changes in the organization of the nucleolus. The cytoplasm of the astrocytes also showed changes including hypertrophy of intermediate filaments and the appearance of an extensive tubular network linking stacks of Golgi cisternae. These morphological changes were accompanied by an increase in GFAP and vimentin expression (53). A stereological analysis of three different regions of the hippocampus in mice ranging in age from 4 to 28 mo found no statistical differences in the numbers of astrocytes or microglia in any of the three areas studied (54). The varying responses obtained in these studies undoubtedly reflect variability in the cellular responses to aging in different regions of the brain and may also indicate that not all

species show identical responses. They also point to differences both between individuals and between groups of animals from different backgrounds.

Similar studies of brain tissue from humans with no signs of neurological disease suggest that only limited changes in cell numbers occur with age. Neuronal cell death is not widespread (55,56), though some regions of the brain such as the hippocampus may undergo greater loss of neurones than other less vulnerable regions (57) and neuron shrinkage and synaptic loss may also occur (58). These changes can, however, be partly offset by growth and elaboration of dendrites in affected areas and such compensatory growth occurs even in aging (>70-y-old) humans (59). Changes in glial cell numbers are similarly limited. There appears to be no overall increase in glial proliferation with age but some reactive gliosis is associated with those regions of the brain where synaptic modification occurs in response to the aging process.

There is no reason to believe that these modest morphological responses to aging would have an effect on responses to brain injury were they not accompanied by a notable upregulation of both astrocyte and microglial cell activation. Studies designed to identify activation of astrocytes during aging show an increase in expression of GFAP with age, which is unrelated to cell numbers (60) but depends upon increased levels of GFAP gene transcription (61,62). Thus, in neurologically normal humans significant correlations between GFAP levels and age have been identified. A study by David et al. (63) showed increased expression of GFAP with age, visible first in the hippocampus, and subsequently, in the entorhinal cortex and isocortex. Levels of expression did not show a steady rise throughout life but increased sharply at ~65-yr-of-age. Similar increases have been shown in other species, for example, in the hippocampus, septum and corpus callosum of the rat (64). Astrocyte activation in aging is accompanied by upregulation of a number of proteins including GFAP, MHC class II, S100 β , monoamine oxidase, superoxide dismutase and $\alpha\beta$ -crystallin. Upregulation does not occur globally throughout the brain: Levels of S100 β increased most markedly in the hippocampus of aging senescence-accelerated mice and paralleled the observed increase in the number of astrocytes expressing GFAP (65).

Microglial activation is also recognized as a common feature of aging brains. A study of differences in antigen expression by microglial cells from young (3–6-mo-old) and aged (24–30-mo-old) rats showed upregulation of complement type-3 receptor, MHC class II, leucocyte common antigen, CD4, and a macrophage antigen recognized by a monoclonal antibody, ED1 (66). A similar result is obtained in monkeys, with microglial cells showing an age-related increase in MHC II expression (67). Both studies showed that enhancement of MHC II expression is largely associated with microglial cells in the white matter. Signals from degenerating white matter induce expression of MHC Class II on microglial cells (68) and thus the upregulation of this molecule in aging brains may be a marker of degenerative changes taking place within the myelinated axon bundles. The upregulation of MHC Class II molecules occurring on both microglia and astrocytes in aged brain may also reflect the integrity of adjacent neurones since, in rat hippocampal slice cultures, interferon- γ only induced MHC class II in regions where there was substantial neuronal damage. Intact neurones prevented astrocyte expression unless channel blockers were used to suppress spontaneous neuronal activity (69).

Increased production of cytokines by activated microglia may also play an important role in the ability of the aging brain to respond to stress or injury. A study of age-

related interleukin-1 α -immunoreactivity (IL-1 α +) in mesial temporal lobe microglial cells of neurologically normal humans indicated that total numbers of activated IL-1 α + cells increased with age. Three types of IL-1 α + cells were identified: Primed, enlarged, and phagocytic, and these were taken to represent three progressive stages of activation. The proportion of enlarged IL-1 α + cells increased threefold whereas phagocytic IL-1 α + cells increased 11-fold in individuals over 60 yr of age. At the same time, high levels of IL-1 α mRNA were found in tissue extracts (70). The effects of IL-1 α include upregulation of neuronal β -amyloid precursor protein expression, activation of astrocytes and promotion of S110 β expression. A similar increase in expression of interleukin-1 β has been found in the hippocampus of aged rats (71). This cytokine has the potential to influence repair processes at a number of levels since, when applied exogenously to the hippocampus, IL-1 β inhibits Ca-influx, protein kinase C and the release of acetylcholine and glutamate. It has also been shown to inhibit long-term potentiation, possibly through the production of reactive oxygen species, which enhance lipid peroxidation and cause neuronal membrane damage (72). Another inflammatory cytokine, IL-6, is overexpressed by brain microglia in the cerebellum, cerebral cortex and hippocampus, but not the hypothalamus, of aged mice (73).

The underlying causes of increased glial cell activation in aging brain are not well understood but since glial cells are known to be particularly responsive to changes in their environment they may include factors that affect brain homeostasis. Among these is a reported reduction in intracellular pH with aging (74) and a reduction in Ca²⁺ channel antagonist receptors on neuronal membranes leading to increased intracellular concentrations of Ca²⁺ (75). The possibility of age-related modulation of blood-brain-barrier permeability suggested by the increased rates of transfer of macromolecules from the blood into the hippocampus and dorsal thalamus that occur in SAM-P8 mice (76) is supported by the observation that specific carrier-mediated transport systems in the blood-brain-barrier of humans, including choline and glucose transfer systems, alter with aging (77). Such changes, coupled with the increased levels of free radicals associated with aging, could impose further strains on the mechanisms responsible for maintaining brain homeostasis.

5. GLIAL RESPONSES TO INJURY IN AGING CENTRAL NERVOUS SYSTEMS

There are strong parallels between glial responses to injury and to aging (Table 2) and it appears that aging induces a constant level of raised reactivity in both microglia and astrocytes. The effect of this on glial responses to lesion are not clear, although a few studies have now been carried out and these suggest an impairment of responses to injury in the CNS of aged, but otherwise healthy individuals. In one such study, the levels of GFAP mRNA were measured in the spinal cord of young (2-mo-old) and older (8 to 17-mo-old) rats. The level increased with age, being 0.4-fold higher in the older group. Sciatic nerve axotomy caused an increase in mRNA levels for GFAP in the young group indicating that astrogliosis was initiated but no response occurred in the older group (78). This suggests that the increased astrocyte reactivity indicated by high levels of GFAP mRNA in older animal prevents further upregulation of GFAP in response to a lesion. Does this occur because age-induced upregulation of GFAP reaches a maximum level above which no further increase in GFAP production is pos-

Table 2
A comparison between the responses evoked by injury and by normal aging in brain tissues

Effects of injury	Effects of age
perturbation of brain homeostasis: changes in ionic flux across cell membranes	changes in brain microenvironment: lowering of intracellular pH
neuronal damage: debris removed by phagocytic activity of microglial cells and invading macrophages	some neuronal loss by apoptosis: sequestration of waste products in microglial cell cytoplasm
release of potentially toxic materials from damaged cells and activated glia	build up of potentially damaging reactive oxygen species and advanced glycation end products
oligodendrocyte death: demyelination may be enhanced by reactive microglia	some loss of oligodendrocytes
rapid activation of microglia and induction of inflammation	increasing numbers of activated microglia present in some brain regions
activation of astrocytes: upregulation of GFAP and immunomodulatory cytokines	increased levels of astrocyte activation marked by increased level of GFAP and morphological changes
upregulation of MHC class II and other immune system antigens	increased expression of MHC class II and other immune system antigens
secretion of IL-6 by microglia: proinflammatory	increased levels of IL-6
TGF- β -1 secreted by microglia cells has immunosuppressive effect	loss of sensitivity to regulatory effects of TGF β -1

sible, or does the upregulation render the CNS less responsive to signals from damaged neurons, particularly in cases like sciatic nerve axotomy where neuronal damage is restricted? It appears that microglial cells do react to this type of sciatic nerve injury in older rats and only the astrocyte response fails (79), indicating that signals from damaged neurones are not suppressed but may be insufficient to activate further astrogliosis against a background of permanently enhanced astrocyte activity.

The fact that in aged animals microglia can continue to upregulate their activity in response to injury although astrocytes cannot could have serious consequences for repair processes. The inflammatory activity of the microglia, which in younger animals is modulated by anti-inflammatory cytokines from reactive astrocytes, will escalate in the absence of astrocyte upregulation. The resulting uncontrolled inflammation will cause increased neuronal loss, demyelination, and oligodendrocyte death. In addition, glial scar formation to isolate the lesion site will be impeded, although ECM molecules and cytokines inducing oligodendrocyte precursor differentiation and migration will not be upregulated. In fact, the number of precursor cells available to replace damaged oligodendrocytes will decline throughout life as cells are withdrawn from the limited

pool of progenitors to effect repair. Thus, depending on the previous history of the individual, oligodendrocyte replacement may be doubly challenged by aging.

An *in vitro* study comparing the behavior of glial cells derived from the brains of young (3-mo-old) and old rats (30-mo-old) shows that both astrocytes and microglial cells from older animals proliferate more readily in culture and that they are less responsive to controlling factors such as GM-CSF and TGF- β 1 (80). However, some astrocyte responses appear to be less affected by aging; in cultured cells derived from aged rats, astrocytes are responsive to neurotrophic signals for many passages although a stage is eventually reached (after ~ 46 passages) when responsiveness decreases and cell homeostasis is impaired (81).

Even in cases where glial cells respond to injury, the responses may be reduced in older animals. A proliferative glial response to deafferentation of the dentate gyrus occurs in both young and aging rats but the older animals (12–26-mo-old) showed a much lower level of increased insulin-like growth factor (IGF-1) mRNA expression in response to the injury, and a reduced capacity for neuronal sprouting (82). The increases in IGF-1 mRNA seen after deafferentation in young rats are associated with microglial cells, so it appears that although microglia in aging individuals can still proliferate in response to injury they may lose their ability to upregulate neurotrophic molecules such as IGF-1. Alternatively, the signaling events that lead to upregulation of IGF-1 in younger animals may be impaired with aging.

The failure of the astrocyte reaction in aged rats is not confined to that species. A study of astrocyte activation in response to ischemic stroke in middle-aged (40–65-yr-old) and elderly (80–101-yr-old) humans showed that reactive astrocytes appeared in the lesion more slowly in the elderly group (5 d post-lesion compared to 3 d) and that the reaction was both less pronounced and of shorter duration (83). Again it is possible to speculate that the reasons for this may depend upon a decreased responsiveness of the aging brain to proliferative and migration-inducing signals from cytokines or a decreased sensitivity to neuronal damage. An age-related decrease in sensitivity to extracellular signals might result from a change in the glial cells themselves or to changes in the brain environment rendering the glial cells less responsive. More research is needed to establish the relative importance of these different elements in the impairment of glial responses to damage that occurs with age but, given the rapid advances that have been made in elucidating glial cell function, the prospects for unraveling the relationships between glial cells, neurons, and brain function in normal aging have never looked more hopeful.

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ATP Signaling in Schwann Cells

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1. INTRODUCTION

Since the early 1970s, strong evidence has been provided that adenosine 5'-triphosphate (ATP) acts as a potent extracellular chemical messenger in cell of many types. It is now well-known that ATP acts as a neurotransmitter at synapses in both the central and peripheral nervous systems. In addition of being a neurotransmitter, ATP and its products of degradation (ADP, AMP, and adenosine) are also known to act as trophic factors by promoting cell proliferation and/or differentiation, stimulation of synthesis and/or release of neurotrophic factors both under physiological and pathological conditions. All these diverse biological effects are mediated via cell surface receptors termed purinoceptors. Purinoceptors of the P₂ type, for which ATP is the physiological ligand, are subdivided into receptors that directly gate ion channels (P_{2X}) and G protein-coupled receptors (P_{2Y}).

Within the peripheral nervous system (PNS), Schwann cells establish and maintain a very intimate relationship with neurons. As ATP is released from virtually all peripheral nerves, neighboring Schwann cells are natural candidates for being modulated by extracellular purines. The recent discovery of the expression of both types of P₂ purinoceptors by Schwann cells brings new insights to neurone-Schwann cell interactions, but how and what for ATP is signaling to Schwann cells is still poorly understood.

This chapter will mainly focus on release of ATP in the PNS and its actions on different P₂ purinoceptors on Schwann cells. We will also discuss putative roles of ATP signaling to Schwann cells.

2. ATP IS RELEASED BY NEURONS OF THE PNS

2.1. *ATP is Released from Peripheral Nerve Terminals*

Holton and Holton (1) and Holton (2) were the first to show that ATP is released during peripheral nerve stimulation. They reported that electrical stimulation of sensory nerves in the rabbit ear evoked release of large amounts of nucleotides in the bloodstream which caused vasodilatation, and that ATP was the nucleotide that most potently induced vasodilatation.

Since this pioneer work, it has become apparent that ATP signaling is ubiquitous within the PNS, ATP being costored and coreleased with other neurotransmitters (see below) by a large variety of peripheral nerves. It has been shown that ATP is released from motor nerves terminals, from some sensory-motor nerves, and from sensory nerves specific for a number of different modalities including mechanoreception, chemoreception, and nociception (3). ATP is involved in the nonadrenergic noncholinergic (NANC) neurotransmission which mediates in part the vagal innervation of the heart (4) and also regulates gastro-intestinal motility (5).

ATP storage and release have also been reported for cultured PNS neurons. This has been shown in chick sympathetic neurons (6), rat superior cervical neurons (7), and rat dorsal root ganglion neurons (8).

2.2. Vesicular Release of ATP

The nucleotide contents of synaptic vesicles of a large variety of peripheral synapses have been closely examined. ATP does not appear to be stored on its own in vesicles, but is costored and coreleased together with other neurotransmitters (9). The nature of the co-transmitters varies considerably with location and species (10) but a general pattern is emerging. ATP is usually costored and coreleased with acetylcholine in motor nerves and parasympathetic nerves. In sympathetic nerves supplying vascular and visceral musculature, ATP is costored and coreleased with noradrenaline. Last, in some sensory-motor nerves ATP co-exists with calcitonin gene related peptide (CGRP) and substance P. Uptake of ATP into vesicles is achieved by a vesicular nucleotide transporter of low affinity (11) which stores additional nucleotides like UTP and GTP.

Elegant studies done at the frog neuromuscular junction showed that motor nerve stimulation *in situ* induced an increase in intracellular calcium in perisynaptic Schwann cells (12) that are mimicked by local application of acetylcholine and ATP (13). Then Robitaille (14) established that neuronal ATP released in the synaptic cleft during neurotransmission was activating two types of purinoceptors (P_{2X}-like and P_{2Y}-like, see **Subheadings 3.1, and 3.2**) expressed by perisynaptic Schwann cells and linked to intracellular calcium signaling.

The mechanism of release of ATP follows some of the general criteria of neurotransmitter release. ATP release is triggered by evoked action potentials, abolished by blockers of voltage-dependent calcium and sodium channels and by withdrawal of calcium from the extracellular medium in cultured chick postganglionic sympathetic neurons (6). In the same study, the authors reported that electrical activity increased the basal efflux of ATP fivefold (respectively 0.15 ± 0.04 pmol ATP mg⁻¹ protein min⁻¹ and 0.82 ± 0.15 pmol ATP mg⁻¹ protein min⁻¹) and that the ATP/noradrenaline ratio in storage vesicles was 1:5.8. ATP is released in quanta from peripheral adrenergic varicosities (15).

Estimations of the intra-vesicle concentration of ATP reveal considerable variance, probably due to methodological limitations, but it is generally accepted that ATP is stored in the hundred-millimolar range (16) although the concentration of the co-transmitter is always several fold higher.

Taken together, all these data are consistent with vesicular release of ATP. However some other results suggest different mechanisms. For example, synaptosomal release

of acetylcholine and ATP from peripheral electromotor synapses of the Torpedo electric organ is differentially blocked by Ω -conotoxin, a voltage-dependent calcium channel blocker (17). Release of ATP and noradrenaline from sympathetic nerves is differentially affected by the electrical pattern of stimulation (6). These data suggest that ATP may be released from neuronal cytoplasmic sources other than synaptic vesicles, although preferential release of one neurotransmitter from a mixture in a vesicle seems to be not necessarily impossible.

2.3. Possible Mechanisms of Nonvesicular Release of ATP

Nonvesicular release of ATP along axons has been postulated by Lyons et al. (18) and more recently substantiated by Stevens and Fields (18a). As a matter of fact, non-vesicular release of other neurotransmitters along central and peripheral axons have been reported in batrachians and mammals. Electrical stimulation triggered release of glutamate and aspartate from peripheral and central frog nerve trunks (19) and release of a neurotransmitter (possibly glutamate or adenosine) in rat optic nerve (20). Acetylcholine is released by depolarization along preganglionic axons of sympathetic nerve trunk of the cat (21). Since axons lack vesicular means of releasing neurotransmitters (22), release mechanisms based on transporters or others transmembrane pathways have to be considered. In **Subheading 2.3.1**, we will present recent data on different possible routes for ATP release in the extracellular medium.

2.3.1. ATP-Binding Cassette Family of Transporters

Members of the ATP-binding cassette (ABC) (*see ref. (23)*) family of transporters are thought to be able to conduct large anions such as ATP. Among the ABC transporter family, the cystic fibrosis transmembrane conductance regulator (CFTR) and the P-glycoprotein have been studied in detail. A new pathway, (NPPB pathway) probably also mediated by an ABC transporter has been very recently identified and will also be presented.

2.3.1.1. CFTR

The CFTR belongs to a superfamily of proteins conducting ions, proteins, and hydrophobic substances. Recent studies have shown that CFTR functions as a protein kinase A-sensitive chloride channel regulated by intracellular ATP (24,25). Loss of CFTR-mediated chloride conductance from the apical plasma membrane of epithelial cells is a primary physiological lesion in cystic fibrosis (26). In addition to its well-known chloride conductance activity, work on human epithelial CFTR, either expressed in mouse mammary carcinoma cells (27) or incorporated into a lipid bilayer (28), has been recently shown that it can mediate release of ATP to the extracellular medium. Whether or not CFTR does possess intrinsic ATP channel activity or triggers release of ATP indirectly remains controversial (25).

2.3.1.2. P-GLYCOPROTEIN

The multidrug resistance (mdr1) gene product, P-glycoprotein is responsible for the ATP-dependent extrusion of a large variety of compounds from cells including multiple cytotoxic drugs. How this efflux transporter functions and what is its physiological relevance remains to be determined (29). P-glycoprotein also regulates a volume-sensitive chloride channel (30) via a protein kinase C-mediated mechanism (31) and functions as an ATP channel (32).

2.3.1.3. NPPB PATHWAY

In a very recent study, Mitchell et al. (33) have identified another route for release of ATP in cultured ciliary epithelial cells. Release of ATP is triggered by the activation by extracellular hypotonicity of a chloride conductance and blocked by the chloride channel blocker 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB). The pharmacological profile of the NPPB pathway excludes CFTR and P-glycoprotein but does not rule out the involvement of other members of the ABC transporter family (34).

2.3.2. Connexins

Gap junction proteins (connexins) are specialized channels formed between cells or different compartments of individual cells which permit the passage of ions, amino acids, second messengers molecules and small metabolites (35). Connexins span two plasma membranes and result from the association of two connexin-hemichannels (connexons). Cotrina et al. (36) recently reported that ATP release by different cell lines (C6 glioma, HeLa, and U373 glioblastoma) was tightly linked to connexin expression (Cx43, Cx32, Cx26). Furthermore, ATP release was facilitated in calcium-free medium which is consistent with the known regulation of the connexin permeability by calcium (37). Then Cotrina et al. (36) suggested that ATP could be released in the extracellular medium through connexons.

2.3.3. Do Such Pathways Exist Within the PNS?

To our knowledge, the detection of members of the ABC transporter family in the PNS has not been reported in the literature, in contrast to the CNS where they are widely expressed by neurons throughout the rat and mouse brain (38) and by astrocytes (39). In contrast to the CNS where connexins are expressed both by neurons and glial cells, connexins in the PNS seem to be only expressed by Schwann cells (40). However a recent study by Dezawa et al. (41) suggest that Cx32 is located between regenerating axons and Schwann cells. It is therefore conceivable that axonal ATP might be released by this pathway during regeneration and could act on neighboring Schwann cells (*see Subheading 5.1.*).

2.4. Fate of Extracellular ATP

By being released either in the synaptic cleft or possibly in the periaxonal space, one can expect, because of the narrowness of these extracellular compartments, that the concentration of ATP could rise up to millimolar levels. However, when present in the extracellular space, ATP is degraded by sequential removal of phosphate groups to give ADP, AMP, and finally adenosine. This process is catalyzed by a series of enzymes (ecto-nucleotidases) located on the extracellular surface of plasma membrane (42).

Two ecto-nucleotidases have been studied in details: The ecto-ATPase which hydrolyses ATP in ADP and the ecto-5'-nucleotidase which hydrolyses AMP giving adenosine. The localization of ecto-nucleotidases has been investigated using enzyme cytochemical techniques (16). In the PNS, ecto-ATPase is mainly localized on the plasma membrane of unmyelinated fibers (43) and also on the surface of nerve terminals of the myenteric plexus (44). The ecto-5'-nucleotidase is found on glial cells (Schwann cells and satellite cells) and also on capillary endothelial cells (16) but has not been detected in peripheral neurons (45,44).

Because biochemical studies of ecto-nucleotidases and determination of their kinetic constants (K_m and V_{max}) have been carried out on synaptosomal preparations or on *Xenopus laevis* oocytes, very little is known about their direct functional role *in situ* in nucleotide degradation at the synaptic compartment. However, the higher potency to ATP itself of weakly hydrolyzable analogs of ATP (e.g., α,β -methylene ATP or ATP- γ -S) in causing contraction of various visceral smooth muscle tissues (46) suggests that endogenous ecto-ATPases are functional *in situ*. By using suramin (a P_2 purinoceptor antagonist which also inhibits ecto-ATPase activity) in the rabbit ear artery, Crack et al. (47) have concluded that the potency of ATP for P_{2X} purinoceptors would be 400-fold higher in the absence of endogenous ecto-ATPase.

At the neuromuscular junction in rat tail artery, the time for clearance by ecto-ATPase of a single quantum of ATP released by a single pulse or short trains (10 pulses) at 50 Hz has been estimated to be less than 100 msec (48). Same experimental measurements of the time of clearance of ATP have not been done for the periaxonal space. However, if we make the assumption than the ecto-ATPase expressed by Schwann cell plasma membranes display the same surface density and kinetic constants than the ecto-ATPase expressed by *Xenopus laevis* (49), it is possible to estimate the time of clearance of ATP in the periaxonal space. Considering that the diameter of an oocyte is 1 mm, its area will be $3.14 \cdot 10^6 \mu m^2$. The V_{max} of the ecto-ATPase is $15.8 \text{ pmoles } P_i \text{ min}^{-1} \text{ cell}^{-1}$ which recalculated and expressed by surface unit gives $8.38 \cdot 10^{-8} \text{ pmoles } P_i \text{ sec}^{-1} (\mu m^2)^{-1}$. Assuming that the width of the periaxonal space is about 10 nm (50), to a Schwann cell membrane area of $1 \mu m^2$ will correspond a volume of periaxonal space of $0.01 \mu m^3$. If the concentration of ATP released in the periaxonal space reaches 1 mM, then the quantity of ATP in $0.01 \mu m^3$ will be $0.01 \cdot 10^{-6}$ pmoles. Therefore the time necessary for the ecto-ATPase to hydrolyse ATP will be around 120 msec.

3. ATP ACTS ON SCHWANN CELLS VIA DIFFERENT TYPES OF P_2 PURINOCEPTORS

The classification and the distribution of purinoceptors has been widely and extensively reviewed in the past few years (9,10,51–53). Basically there are two main families of purinoceptors, P_1 purinoceptors for which adenosine is the natural ligand and P_2 purinoceptors for which ATP is the natural ligand. ATP responses reported in Schwann cells mainly involved P_2 purinoceptors and we will therefore focus on this family (see **Subheading 3.1.1**). The P_2 purinoceptor family is divided into two main subtypes based on their receptor signal transduction mechanisms: P_{2X} purinoceptors are ligand-gated ion channels (ionotropic purinoceptors) while P_{2Y} purinoceptors are G-protein coupled receptors (metabotropic purinoceptors). Schwann cells express purinoceptors of both subtypes.

3.1. P_{2X} Purinoceptors

3.1.1. General Features

Up to now, seven P_{2X} purinoceptors ($P_{2X1} \dots P_{2X7}$) have been cloned and expressed in *Xenopus* oocytes (53). P_{2X1} to P_{2X6} purinoceptors are ATP-gated ion channels selectively permeable to cations (Na^+ , K^+ and Ca^{2+}). Because ATP directly gates the cationic channel, the onset of the current is rapid (within 10 msec). The transmembrane influx

of cations results in a membrane depolarization and an increase in the intracellular calcium concentration ($[Ca^{2+}]_i$). The membrane depolarization leads to the secondary activation of voltage-dependent calcium channels which reinforces the intracellular calcium signal. The P_{2X7} purinoceptor is somewhat special as it has been identified as a cytolytic purinoceptor (54). Depending on the concentration of extracellular ATP and the length of stimulation, P_{2X7} purinoceptors form a nonselective cationic pore. These pores carry molecules with a molecular mass up to 900 Da, and may lead to necrosis or apoptosis of the cell (55).

3.1.2. P_{2X} Purinoceptors Expressed by Schwann Cells

Robitaille (14) was the first to report that Schwann cells expressed P_{2X} purinoceptors. He studied perisynaptic Schwann cells at the frog neuromuscular junction which possess a P_{2X} purinoceptor activated by ATP released during synaptic transmission. Its activation by ATP or L-AMP-PCP (a nonhydrolyzable ATP analog with a high affinity for P_{2X} purinoceptors) triggers an increase in $[Ca^{2+}]_i$ which is dependent on external calcium and strongly reduced by L-type calcium channel blockers. Robitaille (14) concluded that the membrane depolarization resulting from a modest influx of calcium through the P_{2X} purinoceptors activated L-type calcium channels and thereby produced a large calcium entry. The pharmacological profile of this response has been not investigated further so the subtype of P_{2X} purinoceptor involved remains to be identified.

Amédée and Despeyroux (55a) first reported the existence of an unusually low affinity ($K_d = 8.4$ mM) ATP-activated mixed current composed of cationic and anionic conductances in Schwann cells cultured from dorsal root ganglia of the mouse. Later using potent agonists (BzATP and ATP^{4-}) and antagonist (α ATP) together with a polyclonal antibody raised against P_{2X7} purinoceptors, Colomar and Amédée (56) clearly demonstrated the expression of a P_{2X7} purinoceptor in mouse Schwann cells. In addition to the well documented formation of a non selective cationic pore, the stimulation of the mouse P_{2X7} purinoceptor leads to the activation of two other associated ionic conductances, that is a Ca^{2+} -dependent K^+ conductance and Cl^- conductance. An ionotropic purinoceptor with electrophysiological and pharmacological characteristics similar to the P_{2X7} subtype has also been identified in the paranodal membrane of rat Schwann cell (57). ATP and BzATP activate a nonspecific cation current and confocal calcium imaging showed a sustained rise in $[Ca^{2+}]_i$ which is exclusively dependent on external calcium.

3.2. P_{2Y} Purinoceptors

3.2.1. General Features

To date five P_{2Y} purinoceptors (P_{2Y1} , P_{2Y2} , P_{2Y4} , P_{2Y6} and P_{2Y11}) have been cloned and firmly identified as members of the P_2 purinoceptor family (53). P_{2Y} purinoceptors belong to the superfamily of receptors that have seven transmembrane domain and are coupled to G-proteins. Binding of ATP on P_{2Y} purinoceptors activates phospholipase C and the formation of inositol 1,4,5-trisphosphate (IP_3) which releases calcium from intracellular stores. Ion channels either regulated by G-proteins or by an increase in the $[Ca^{2+}]_i$ can also be activated following P_{2Y} stimulation. Because of

the involvement of second-messenger systems, the delay between the stimulation of the receptor and the onset of the cellular response is longer (> 100 msec) than that of P_{2X} purinoceptors.

3.2.2. P_{2Y} Purinoceptors Expressed by Schwann Cells

Lyons et al. (58) were the first to report the expression of P_{2Y} purinoceptors by Schwann cells cultured from rat sciatic nerves. ATP induced a transient increase in $[Ca^{2+}]_i$. This response disappeared within 4 d in vitro, but was restored by culturing the cells in the presence of cAMP analogs. In the subsequent work, Lyons et al. (18) reported that the ATP-mediated calcium response of the Schwann cells could also be maintained or restored by contact with neurons. According to Ralevic and Burnstock (53), the sequence of relative agonist potency (ATP=2MeSATP=ADP β S; adenosine and UTP ineffective) of this receptor was similar to those described for the cloned P_{2Y1} purinoceptors.

Since then, ATP-responses mediated by P_{2Y} purinoceptors have been reported in other Schwann cells. P_{2Y} purinoceptors responses have been reported in cultured Schwann cells from rat and rabbit sciatic nerves (59), mouse dorsal root ganglia (60), rat dorsal root ganglia (61) and on Schwann cells acutely dissociated from the neuro-electrocyte junction in skate (62). In all these studies, the ATP signaling was invariably associated with release of intracellular calcium from IP_3 sensitive calcium stores.

In most cases an adequate pharmacological characterization and/or molecular identification of the P_{2Y} purinoceptors subtype involved is lacking even though it seems to be an important issue for axon-Schwann cell interactions. For example, the Schwann cell phenotype (i.e., myelinating vs nonmyelinating) and the molecular identity of the P_{2Y} subtype are linked. Myelinating Schwann cells from human sural nerves, rat ventral roots, and vagus nerves express a P_{2Y2} -like purinoceptor, whereas nonmyelinating Schwann cells express a P_{2Y1} -like purinoceptor (63). Moreover, the P_{2Y2} -like purinoceptor is preferentially expressed in the paranodal region of myelinating Schwann cells (64).

4. EFFECTS OF EXTRACELLULAR ATP ON SCHWANN CELLS

4.1. Change of Membrane Potential

Modulation by ATP of membrane potential may involve different subtypes of P_2 purinoceptors. Basically ATP can either gate directly nonselective cationic channels (P_{2X} -like purinoceptors) and therefore allow the influx of Na^+ , K^+ , and Ca^{2+} which will depolarize the cell, or by acting on G-protein coupled P_{2Y} -like purinoceptors, release calcium from intracellular stores which may in turn trigger the activation of calcium-dependent ion channels. The resulting change in membrane potential may lead to the secondary activation of voltage-dependent ion channels.

Such mechanisms or at least part of these sequences of events are known for neurons and glial cells of the CNS (65,66) but are still hypothetical for Schwann cells.

Amédée and Despeyroux (55a) reported that in Schwann cells cultured from dorsal root ganglia of the mouse, ATP induced a mixed current composed of a cationic (K^+ and anionic (Cl^-) conductances. This current by depolarizing the Schwann cell up to -40 mV may activate voltage-dependent Ca^{2+} channels (67).

ATP (1 mM) induces a nonspecific cation current in cultured rat Schwann cells from spinal roots (57) via an ionotropic purinoceptor of characteristics similar to those of P_{2X7} -like purinoceptors.

4.2. Modulation of Intracellular Calcium

Calcium ions play an important role in regulating very many glial functions both on short time scale (release of neuroactive molecules, ion regulation...) through the activation of protein kinases, protein phosphatases, phospholipases, adenylate cyclases, and gating of ion channels, and on a longer time scale (cell cycle, proliferation, and differentiation...) through gene expression (68–70).

Intracellular free calcium concentration whatever the cell type, is tightly regulated by several mechanisms. It is sequestered in intracellular stores and complexed to calcium binding proteins. Calcium fluxes occur through different pathways (voltage-dependent calcium channels, ionotropic receptors, store-operated channels...) and its extrusion is carried by specialized proteins (Ca-ATPase, Na/Ca exchanger). In Schwann cells, resting $[Ca^{2+}]_i$ varies from 35 to 100 nM (59,62,71,72), a value within the range reported for glial cells of the CNS (30–40 to 200–400 nM,[73]).

Modulation of $[Ca^{2+}]_i$ triggered by ATP stimulation is dependent on intracellular and/or extracellular sources of calcium in Schwann cells. The ratio of both sources varies among the species. In cultured adult rat and rabbit Schwann cells, ATP (100 μ M) triggers a fast monophasic transient increase in $[Ca^{2+}]_i$ which is independent of the presence of external calcium and fades rapidly upon repeated stimulation (59).

In contrast, in cultured mouse Schwann cells, ATP (1 nM–100 μ M) triggers a biphasic increase in $[Ca^{2+}]_i$: an early fast transient increase followed by a plateau phase which slowly declined to resting level (60). The plateau phase is suppressed by removal of external calcium, whereas the early fast transient increase is unaffected by such removal. Similar biphasic ATP responses have been reported in perisynaptic Schwann cells at the neuroelectrocyte junction in skate (62).

The amplitude of the $[Ca^{2+}]_i$ increase varies with the concentration of ATP used and the species. In cultured rodent Schwann cells, ATP (100 μ M) increases $[Ca^{2+}]_i$ two- to fourfold (59) in skate perisynaptic Schwann cells, ATP (50 μ M) increases $[Ca^{2+}]_i$ sevenfold (62).

From all these different studies, it can be concluded that the first phase is due to the release of calcium from intracellular calcium stores following stimulation of P_{2Y} -like purinoceptors leading to the generation of IP_3 . The secondary plateau phase probably involves extracellular calcium influx activated by the depletion of intracellular stores ([capacitative calcium entry],[74]). This influx is thought to be necessary for the refilling and/or maintenance of IP_3 mobilized intracellular calcium stores.

4.3. Release of Neurotransmitters

The release of neurotransmitters by glial cells of the CNS is well-known to be a calcium-dependent process (75). Because Schwann cell calcium levels are modulated by ATP and other neuroligands, this has prompted studies to investigate the possibility of a neuroligand evoked calcium-dependent release of neurotransmitters by Schwann cells.

Acetylcholine was probably the first neurotransmitter found to be released by Schwann cells. Katz and Miledi (76) reported the occurrence of spontaneous miniature

end-plate potentials (min.e.p.p.s) after complete denervation of amphibian end-plates and suggested that these potentials were due to acetylcholine release by Schwann cells. Later, Dennis and Miledi (77) showed that electrically stimulated denervated Schwann cells released acetylcholine in a nonquantal form. In giant axons of the squid, Lieberman et al. (78) have reported that nerve stimulation caused Schwann cell hyperpolarization. They suggested that glutamate released by axons caused Schwann cell depolarization which in turn triggered the release of acetylcholine and caused by an autocrine loop Schwann cell hyperpolarization. Although these observations have not been substantiated in other molluscan species and need further confirmation, the calcium permeability of NMDA receptors and their presence in squid Schwann cells (79) make possible that a glutamate-mediated $[Ca^{2+}]_i$ increase in Schwann cells mediates acetylcholine release.

In mammalian Schwann cells, recent work has clearly established the existence of a neurotransmitter-evoked calcium dependent release of neuroligands. Bradykinin is released in the PNS in response to trauma (80). In rat Schwann cells, bradykinin (10 nM) increased $[Ca^{2+}]_i$ sevenfold (from 102 to 747 nM) which caused the release of glutamate and aspartate (71). In the same preparation, ATP (100 μ M), acting on P_{2Y} purinoceptors, also released glutamate and aspartate (61). Glutamate and aspartate release was due to mobilization of IP_3 sensitive calcium stores. Furosemide (5 mM), a chloride cotransport inhibitor blocked ATP-induced glutamate release. The calcium-dependent release mechanisms remain to be determined, but Schwann cells do not express synaptic proteins (synaptophysin and synaptotagmin) characteristic of neuronal transmitter release (71) which suggest that the release mechanism is nonvesicular.

4.4. Cell Death by Apoptosis

It has been well-known for almost 20 yr that high concentrations of extracellular ATP cause cell death in many different cell types including glial cells (81). However, it is only very recently that the membrane receptors involved in cell necrosis and/or apoptosis have been characterized and cloned. Surprenant et al. (54) cloned from rat brain the P_{2X7} purinoceptor and identified it as a cytolytic purinoceptor for extracellular ATP. P_{2X7} purinoceptors have been reported to mediate apoptosis of activated mouse thymocytes (82). The intracellular pathways activated following P_{2X7} purinoceptor stimulation are still poorly known, but there is no doubt that the major perturbation in the intracellular ion homeostasis resulting from the gating of the nonselective pore plays an important role. There is no doubt that the activation by ATP of different caspases lead to cell apoptosis. ATP activates caspase 1 (IL-1 β converting enzyme) in mouse microglial cells (83), caspase 3 and caspase 8, both leading to the apoptosis of microglial cells from the mouse cell line N13 (84). Interestingly, the activation of caspase 1 triggers apoptosis in fibroblasts (85).

The potent ligand for P_{2X7} purinoceptor is known to be the tetraanionic form of ATP (ATP^{4-}). As in physiological solutions, ATP is almost exclusively coordinated to calcium and/or magnesium (86), ATP^{4-} represents only 1–5% of total ATP which may explain the high concentrations of ATP (mM to ten-mM range) needed to activate the receptor. However, bearing in mind that cytoplasmic ATP concentration is in the same range (5–10 mM, [81]), it is plausible that neural ATP released during pathological con-

ditions could reach concentrations high enough to induce necrosis and/or apoptosis of surrounding Schwann cells.

5. PUTATIVE ROLES OF ATP SIGNALING IN SCHWANN CELLS

5.1. *Trophic Actions of Extracellular ATP on Schwann Cells*

The trophic actions of extracellular ATP in the PNS are poorly known. However, some of the purinoceptors expressed by Schwann cells have been reported to mediate mitogenic and morphogenic effects of ATP on astrocytes in the CNS (87,88). This is the case for P_{2Y1}-like and P_{2Y2}-like purinoceptors which are linked to the activation of a mitogen-activated protein kinase (MAPK) in rat cortical astrocytes (89). Activation of the MAPK pathway leads to the phosphorylation of transcription factors which bind to the serum responsive element (SRE) sequence on DNA and trigger changes in gene expression (90).

The activation by extracellular ATP of the MAPK pathway has not been shown directly in Schwann cells. However, Mutoh et al. (91) reported that the MAPK pathway in Schwann cells was activated by changes in the intracellular cAMP levels. Application of 8-bromo cAMP (a cell permeable derivative of cAMP) had effects that depended on the concentration and the length of the stimulation: Schwann cells were driven to a proliferative state by brief stimulation with low concentrations or to a differentiated state by prolonged stimulation with higher concentration. Hence since the P_{2Y1}-like purinoceptor and to a lesser extent the P_{2Y2}-like purinoceptor decrease cAMP levels in rat Schwann cells (72), activation of these receptors is expected to control the switching of the cells between proliferation and differentiation.

5.2. *Schwann Cell Apoptosis During Physiopathological Conditions*

Apoptosis, also known as programmed cell death, is a highly orchestrated cell suicide involving specific cysteine proteases (caspases) which lead to nuclear fragmentation and formation of membrane-packaged cellular debris (apoptotic bodies) that are phagocytosed by macrophages or other surrounding cells (92). Apoptosis plays a major role not only in the developing nervous system but also occurs at adult stages of cell life (93). Neuronal cell apoptosis has been extensively studied during the last decade and it is generally believed that supernumerary neurons which are initially generated, die by apoptosis during normal development to match their number to the requirements of the neuronal circuitry.

Because glial cells are intimately associated with neuronal cells and participate actively in the development of the nervous system, it was therefore of interest to study glial cell apoptosis. Apoptosis has been reported for rat oligodendrocytes in the developing optic nerve (94) and rat astrocytes during the development of cerebellum (95).

5.2.1. *Schwann Cell Apoptosis During Development*

Schwann cells die by apoptosis during normal development, of ventral and dorsal nerve roots in the chick embryo (96) and rat sciatic nerve (97). The mechanisms responsible for the developmental apoptosis of Schwann cells are not completely clear, but neuronal derived trophic factors of the neuregulin family (98) seem to reduce, at

least in part, the Schwann cell apoptosis. This has been shown in the developing rat sciatic nerve, where Schwann cell apoptosis is markedly reduced in vivo by injection of neuregulin in newborn rats nerves (97).

A contribution by purinoceptors to the regulation of apoptosis of Schwann cells during peripheral nerve development has not yet been reported but appears plausible. The growth cones of developing neurons are known to release neurotransmitters. This has been shown in vitro for mammalian CNS (99) and PNS (100) neurons. It is therefore conceivable that like other transmitters, ATP could be released from growth cones and/or along axons of peripheral neurons during development and modulates apoptosis of surrounding Schwann cells.

5.2.2. Schwann Cell Apoptosis During Pathological Conditions

During the Wallerian degeneration which follows experimental or traumatic peripheral nerve transection, the distal stump (transected axon and surrounding Schwann cells) undergo major cellular events (101). Axonal cytoskeleton and axoplasm disintegrate and are converted into granular and amorphous debris, while the vast majority of Schwann cells throughout the distal stump rapidly (in a few hours) undergo apoptosis. This has been shown in the chick embryo (96), rat sciatic nerve (97,102), and rat neuromuscular junction (103).

Because ATP is massively released when cells are damaged (82) it is very likely that it could trigger apoptosis of surrounding Schwann cells. However, in such pathological conditions, loss of neuronal trophic factors also favours Schwann cell apoptosis. For example, Schwann cells at the developing rat neuromuscular junction are rescued in vivo from axotomy-induced apoptosis by glial growth factor (103) one of the members of the neuregulin family (98).

5.3. Schwann Cell Modulation of Neuronal Functioning

Extracellular ATP induces a calcium-dependent release of aspartate and glutamate from cultured Schwann cells (61). In the CNS of mammals, glutamate is the major excitatory neurotransmitter and mediates neuron-glia calcium signaling (20,104,105). One consequence of neuron-glia calcium signaling is the increase or the decrease of the neuronal synaptic efficacy (106). For example, an increase in the synaptic efficacy may be achieved by the calcium dependent activation of astrocytic phospholipase A₂ which produces arachidonic acid and inhibits glutamate uptake.

As peripheral synapses are devoid of glutamate receptors, the existence of this modulatory mechanism is very unlikely in the PNS. However, cytotoxic effects of glutamate and aspartate have been reported in rat sciatic nerve (107) which suggest that at least some peripheral axons can respond to extracellular glutamate.

As in astrocytes, where glutamate induces the release of another neurotransmitter (GABA) through an autocrine pathway (108), glutamate/aspartate release from Schwann cells could stimulate the release of another neurotransmitter which could act on neurons. A glutamate/acetylcholine autocrine/paracrine pathway has been suggested for Schwann cells of the giant axon of the squid (*see Subheading 4.3.*). Figure 1 summarizes some of the possible actions of ATP on Schwann cells in physiological and pathological conditions.

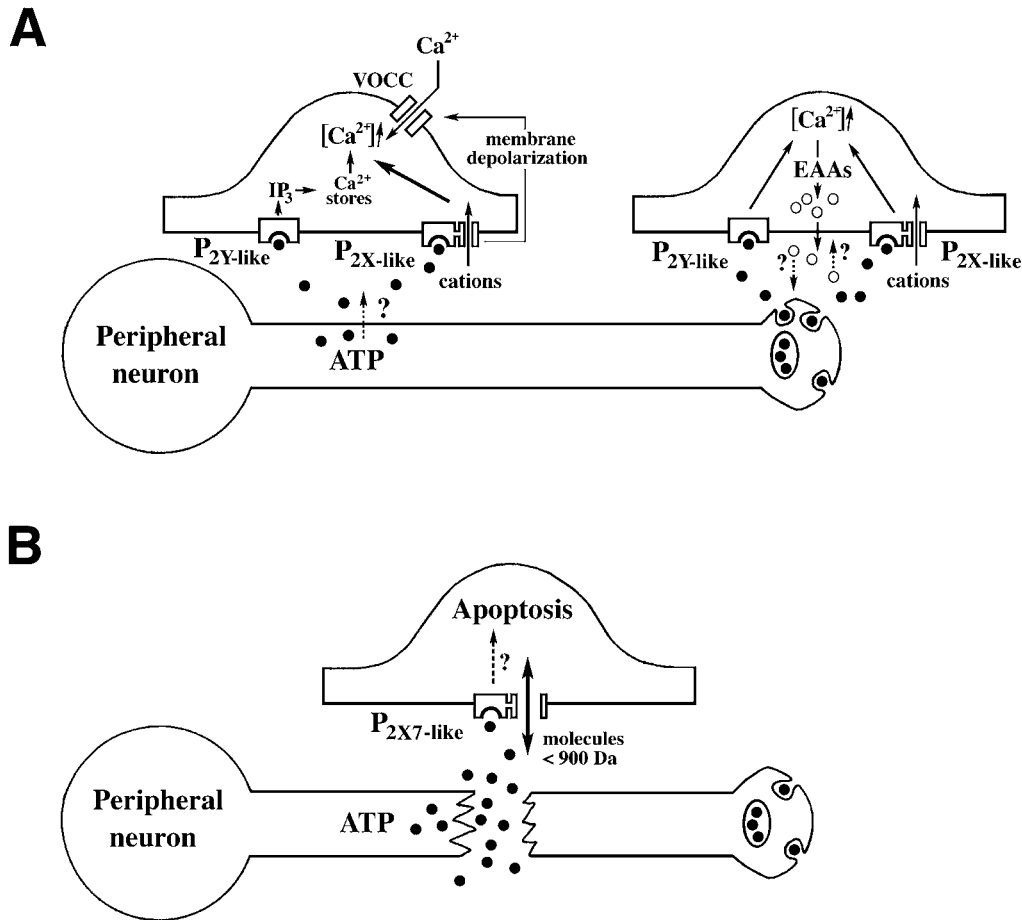


Fig. 1. Scheme of ATP signaling in Schwann cells under physiopathological conditions. **A.** In physiological conditions, ATP is released at synapses and may be released along the axon. For sake of clarity, membrane and intracellular mechanisms leading to an increase in $[Ca^{2+}]_i$ have been detailed in the left hand Schwann cell and calcium-dependent release of EAAs in the right hand Schwann cell. **B.** In pathological conditions, ATP is massively released by lesioned axon and by activating P_{2X7} -like purinoceptor may trigger Schwann cell apoptosis. VOCC: voltage operated calcium channel; EAAs: excitatory amino acids.

5.4. Secretion of Cytokines

Cytokines are pleiotropic signaling peptides which act through paracrine and autocrine networks. They are secreted not only by inflammatory and immune system cells, but also by accessory immune cells such as glial cells of the CNS (astrocytes, oligodendrocytes, and microglia) (109).

Schwann cells have cellular and humoral immune functions (110). They can process exogenous antigens and express MHC molecules (cellular immune functions) but also they synthesize and secrete a number of proinflammatory cytokines including IL-1, IL-6, IFN- α and TNF- α as well as immunoregulatory cytokines including IL-10 or TGF- β (111). In the case of mouse microglial cells (84) and human macrophages (112) release

of IL-1 β is stimulated by ATP. The ATP binds to a P_{2X7}-like purinoceptor and activates IL-1 β converting enzyme (ICE) which converts IL-1 β from its inactive precursor to its biologically active form.

For those Schwann cells which express P_{2X7}-like purinoceptors, their activation may lead to the activation of ICE. This could be a pathway leading to the synthesis and/or secretion of IL-1 β . The consequences are probably quite complex since the same cytokine, i.e., IL-1 β , can stimulate either the immune response or serve as growth or protective factors. For example, IL-1 β induces synthesis of nerve growth factor by Schwann cells (113) which stimulates neuronal repair.

6. CONCLUSION

In this chapter we have highlighted ATP as a neural signaling molecule in the PNS by examining neuronal sites of storage, pathways of release, as well as known and putative effects on surrounding Schwann cells. ATP has a wide range of effects on Schwann cell physiology. It may affect the Schwann cell differentiated/proliferative state by interacting with gene expression through cAMP level and MAPK pathway and/or the synthesis and release of cytokines. By evoking release of excitatory amino acids from Schwann cells, ATP signals to Schwann cells which in turn could signal to neurons (paracrine loop) and/or to Schwann cell itself (autocrine loop).

Most of the time, the response to ATP involves changes in the Schwann cell [Ca²⁺]_i. How does ATP induce such diverse responses in Schwann cells? Some specificity is given by ATP causing either an inhibition of adenylate cyclase via the stimulation of P_{2Y1}-like purinoceptors or an increase in [Ca²⁺]_i. In the latter case, further specificity probably results from the spatiotemporal pattern of the increase in [Ca²⁺]_i. For example the increase in [Ca²⁺]_i may be fast and transient when it is only due to release of calcium from intracellular stores, while it will be slow and sustained when capacitative calcium entry is involved. Last, because ATP is released under different circumstances and in different extracellular spaces, it is likely that the extracellular concentration varies and therefore the subtype of purinoceptor activated may be different. For example, the activation of P_{2X7}-like purinoceptors will require higher concentrations than activation of P_{2Y}-like purinoceptors. Such high concentrations of ATP are likely to be reached during pathological conditions.

By the diversity of its actions, ATP appears to be one of the most powerful signaling molecules involved in neuro-glial interactions in the PNS.

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III

NEUROTROPHINS, GROWTH FACTORS, AND NEUROHORMONES IN AGING AND REGENERATION

Gliososis Growth Factors in the Adult and Aging Rat Brain

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1. INTRODUCTION

Gliososis is a response of glial cells (consisting usually in hypertrophy, migration, proliferation, and phenotypic changes) to various types of neural tissue perturbations. This glial response occurs naturally in aged animals. Notwithstanding the nature of the trauma, the glial response is more or less stereotypical. A reproducible succession of cellular and molecular events develops, coordinated by intercellular signaling in which growth factors and extracellular matrix seem to play a major role. Gliososis can occur in any part of the central nervous system (CNS). Prominent responses that follow brain trauma include the activation of microglia, recruitment of blood-derived macrophages, and astroglial reactivity. Occasionally, depending on the zone of gliosis, other cell types can be activated like ependymal cells, endothelial cells, meningeal cells, and Schwann cells. The main aspect of localized gliosis is the elaboration of a glial scar which forms a physical and biochemical barrier preventing axonal regeneration and remyelination. In addition to this physical repair, many data suggest that gliosis contributes to nerve survival and to immunological protection.

2. GLIOSIS RESULTING FROM BRAIN INJURY IN THE ADULT RAT

2.1. Triggering of Gliosis

Gliososis can be triggered by a great variety of perturbations of the nervous system:

1. Physical injury which can be a stab-wound, possibly associated with heating, freezing or chemical attack, or a physical shock without wound;
2. Infection by external organisms;
3. Various diseases which can affect the CNS such as ischemia, seizures, neurodegenerative diseases, autoimmune diseases or tumors. It seems that the primary event triggering gliosis is a destruction of neurons, but also of oligodendrocytes, astrocytes or other cells. Demyelination is also able to induce gliosis. In order to understand the mechanisms of gliosis or of CNS degenerative diseases, and possibly to prevent them or attenuate their effects, the level of various molecules can be modulated in the CNS or in some parts of it. Some of them will trigger gliosis.

4. The two main ways to achieve this task are: Injection of substances, locally, or systemically when they are able to pass the blood-brain-barrier or to break it, such as growth factors, and cytotoxic molecules;
5. Overproduction or defect of molecules normally present in the CNS, for example by the use of transgenic mice, of mRNA antisense strategies or of immunochemical methods.

2.2. Cells Involved in Gliosis

Gliosis was first defined by the activation of astrocytes. However, it appears that microglia are necessary for astrogliosis to develop (1). Hypertrophy of microglia starts 24 h after injury, and after 2–3 d, they begin to proliferate and to migrate toward the site of the injury. These cells secrete cytokines and factors which can be involved in inflammation, immune defense, neuron protection or regulation of astrocyte activation (2). Within 24 h after cerebral injury, the level of glial fibrillary acidic protein (GFAP) and vimentin (3) increases in astrocytes with an increase of their volume and of the number and length of their processes. GFAP is involved in the mechanism of this activation. Later, they migrate and proliferate, produce extracellular matrix and form a scar composed of cells associated to this matrix. The expression of many proteins is upregulated; nestin, various cytokines, growth factors, carbonic anhydrase, and L-type Ca^{2+} channels. The Ca^{2+} S-100 protein present mainly in astrocytes is increased after aspiration of the occipital cortex and participates in the trophism and plasticity of the injured visual pathway (4). Astrocytes can elicit other effects through the release of growth factors and particularly neurotrophic factors which will help the survival of the damaged neurons.

Recent data suggest that oligodendrocytes are involved in gliosis. There is a swelling of their nucleus and cytoplasm and an increase in number and size of their processes. An increase in the number of oligodendrocytes and an enhanced expression of 2', 3'-cyclic nucleotide phosphohydrolase (CNP) and myelin basic protein (MBP) has also been reported. Ependymal cells can also be involved in gliosis. In response to spinal cord injury, their proliferation increases dramatically to generate migratory cells that differentiate into astrocytes and participate in scar formation (5). The migration and proliferation of ependymal cells appear to depend on growth factors like EGF, PDGF, TGF beta, and thrombin (6). Meningeal cells may invade the lesion and contribute also to the formation of scar.

3. GROWTH FACTORS INVOLVED IN ADULT BRAIN GLIOSIS

This chapter describes which factors are released in gliosis, and when known, in which cells they are produced, on which cells they elicit their main effects, what are these effects, how their expression is regulated, and what are their functions.

3.1. Fibroblast Growth Factor-2 (FGF-2)

FGF-2 is an ubiquitous factor found at a high concentration in adult brain. It elicits pleiotrophic effects on all the nerve cells. In normal rat brain, it is localized in astrocytes, in selected neuronal populations, and occasionally in microglial cells. FGF-2 protein and mRNA are found increased in astrocytes at the site of focal brain wounds (7) and after ischemia, convulsive seizures or kainic acid injection (8), and sometimes, to less extent, in neurons and microglia (9). Reactive gliosis can be stimulated by injec-

tion of FGF-2 in injured rat brain (10–12). Expression of FGF receptor-1 (FGFR-1) increases on astrocytes adjacent to the wound cavity by day 2–7 postlesion until day 10 and decreases to control values by day 28. Thus, through autocrine or paracrine mechanisms FGF-2 could be an early response of reactive astrocytes and it may act on this reactive astrogliosis by stimulating the proliferation, hypertrophy, and migration of astrocytes. FGF-2 also promotes the synthesis and release of extracellular matrix (ECM) (tenascin C) and growth factors, such as, neurotrophic factors (NTFs). In the early phase following brain injury, cytokine-activated astrocytes can rescue the damaged neurons and contribute to the process of axonal outgrowth and synaptogenesis via NTFs and other molecules. After a moderate contusion of rat spinal cord, administration of FGF-2 enhances functional recovery and tissue sparing. After 6 wk, astroglial and microglial cell immunoreactivity was as in controls (13). Since FGF-2 activates quiescent microglia and enhances their proliferation *in vitro*, FGF-2 released by astrocytes should contribute to this activation. In astrocytes, FGF-2 and FGFR can be stimulated by interleukine-1 (IL-1) secreted by activated microglia and by free radicals produced by invading neutrophils. FGF-2 elicits neuroprotective or neurotrophic effects on neurons, directly or indirectly by inducing the expression of neurotrophic factors in astrocytes.

Fibroblasts genetically engineered to produce FGF-2 and implanted in the striatum can protect the nigrostriatal dopaminergic system and may be useful in the treatment of Parkinson's disease (14).

3.2. Platelet-Derived Growth Factor (PDGF)

In normal rats, PDGF B-chain is preferentially expressed within neural cell bodies in the cortex, hippocampus, and cerebellum. An increase in PDGF expression has been observed in various traumatic conditions in the CNS such as after human brain abscess or in proliferative retinopathies (6) or after mechanical injury in rat brain (15). *In vitro*, astrocytes produce A and B chains. Moreover, TGF- β 1, TNF- α , and IL-1 β are able to stimulate astrocyte PDGF B production. In human brain abscess, PDGF immunoreactivity was found in glia, and also in endothelial cells and fibroblasts (16). An enhanced expression of PDGF was also found in macrophages on days 3 and 4 after mechanical lesion (15). PDGF is a strong mitogen for astrocytes *in vitro*. PDGF-AA increases the number of retinal astrocytes and these cells promote the survival of endothelial cells as well as their expression of barrier characteristics (17). PDGF-BB has been shown to exert neuroprotective action on various types of neurons like GABAergic interneurons.

3.3. Transforming Growth Factor- β (TGF- β)

There are three forms of TGF- β (1, 2, and 3) and two types of receptors. All are present in the CNS. Although *in vitro* astrocytes display these three isoforms, neurons express only TGF- β 2. Both type I and type II TGF- β receptors are present in cortical neurons and astrocytes *in vitro*. Thus, TGF- β may act as autocrine and paracrine signals between neurons and astrocytes in the CNS. TGF- β 1 in the rat brain increases after injury and inhibits astrocyte proliferation (18). At days 1–3 following ischemia TGF- β and their receptors are upregulated in the perifocal neurons, reactive astroglial cells, endothelial cells and macrophages (19). Astroglial overproduction of TGF- β 1 in transgenic mice triggers a pathogenic cascade leading to AD-like cerebrovascular

amyloidosis, microvascular degeneration, and local alterations in brain metabolic activities (20). The production of TGF- β 1 is stimulated by IL-1 in astrocytes in vitro. TGF- β inhibit the proliferation of microglia. In vitro, TGF- β 1 promotes survival of rat spinal cord motoneurons and dopaminergic neurons. It protects cultured neurons from damage. The neuroprotective action of TGF- β 1 is mediated by the protease inhibitor PAI-1 produced by astrocytes and possibly by NGF in vivo. Treatment of cerebral wounds with anti-TGF- β 2 antibody leads to a marked attenuation of all aspects of CNS scarring (21).

3.4. Epidermal Growth Factor (EGF) Family

Three members of the EGF family are present in the nervous system, EGF, TGF- α and heparin binding (HB)-EGF. They share the same receptor (EGFR). TGF- α and EGFR produced by neurons and glia, play an important role in the development of the nervous system. Following different CNS traumas, EGFR appears mostly within and around the damaged areas, in reactive astrocytes (22) as well as in reactive microglial cells and released after injury. EGF is not a mitogen for microglia, but rather a chemoattractant, so it may serve to direct microglial cells to the lesion site. Moreover, since EGF is secreted by activated microglia themselves in vivo, it may act as an autocrine modulator of microglial cell function (23). Synthesis of TGF- α , HB-EGF and EGFR in reactive astrocytes following CNS traumas suggests that they play a role in the development of astrogliosis. They are involved in mediating glial-neuronal and axonal-glial interactions and participate in injury associated astrocytic gliosis (24).

The effect of TGF- α has been examined by the use of transgenic mice bearing the human TGF- α cDNA. These mice display enhanced GFAP and vimentin protein levels in brain. Thus enhanced TGF- α synthesis is sufficient to trigger astrogliosis, whereas microglia are unaffected (22). Moreover, a soluble astrocyte mitogen inhibitor is reduced after injury. Intracerebral injection of antibody against it causes the appearance of reactive astrocytes. So, this inhibitor may play a key role in the control of glial cell division in both normal and injured brain. In addition to their role in gliosis, factors of the EGF family also exert neurotrophic activity. EGF extends the survival of cultured neurons, facilitates neurite outgrowth, and prevents neuronal damage caused by various cytotoxic compounds.

3.5. Insulin-Like Growth Factors (IGFs)

This is a family of three factors, insulin, IGF-1, and IGF-2. Six specific IGF binding proteins (IGFBPs) have been identified, which are able to transport and/or modulate the action of IGFs. IGF-1 receptor is ubiquitous. IGF-1 is present mainly in neurons and stimulates the proliferation and survival of neurons and oligodendrocytes. Intact animals showed no detectable IGF-1 immunoreactivity in astrocytes. IGF-2 mRNA expression is observed in the choroid plexus, meningeal membranes, and in blood vessel endothelium. Several observations suggest a role for IGFBPs in targeting the neuroprotective actions of IGF-1. Following electrolytical lesion in rat hippocampus or unilateral hypoxic-ischemic injury, there is an increase in secretion of IGF-1 at the lesioned site (25). IGF-1 may have a role as a neuroprotectant for surviving neurons and a signal for local neuronal sprouting, as well as in reactive astrogliosis. After partial deafferentation of the cerebellar cortex by 3-acetylpyridine injection, continuous

infusion of IGF-1 significantly decreases reactive gliosis while IGFR1 antagonist or IGF-1 antisense increase it. Several authors have shown that IGF-1 is a survival factor, particularly after ischemia, for hippocampal and cortical neurons. Some data provide experimental evidences which suggest that IGF and BDNF may exert their potential neuroprotective effects via regulation of NOS activity (26). IGF-2 has been shown to be secreted within the injured rat brain. During the acute phase, the secretion of IGF-2 from the choroid plexus into the CSF is upregulated and IGF-2 is complexed to IGFBP-2 to be transported to the wound. In the chronic phase of the injury response, IGF-2 reasserts itself to a predominantly autocrine/paracrine role restricted to the mesenchymal support structures, including the glia limitans, which may help to reestablish and maintain tissue homeostasis. Reactive IGF-2 is found in microglia and astrocytes (27). IGF-II receptors were found widely in all neuronal regions and plexus choroids but were not detected in any of the astroglial plaques (26,28).

3.6. Neurotrophins (NGF, BDNF, NT-3, GDNF)

3.6.1. Nerve Growth Factor (NGF)

NGF is a neurotrophic factor for sympathetic and sensory neurons as well as for basal forebrain cholinergic neurons in the brain. In the mature mammalian brain, NGF expression is restricted to neurons. However, astrocytes activated by various cytokines released after brain injury, produce a significant amount of NGF in vitro and in vivo (29). In the early phase following brain injury, these cytokine-activated astrocytes rescue the damaged neurons via NGF and other biologically active molecules (30). The increase in NGF after CNS trauma is directly mediated through IL-1 beta and IL-6. Other cytokines like IL-4, IL-10, and IFN gamma induced the secretion of NGF by activated astrocytes and IL-1 and IL-4 synergized with lipopolysaccharide (LPS) and TNF- α in this effect.

3.6.2. Brain-Derived Growth Factor (BDNF)

BDNF exerts neuroprotective as well as neurotrophic roles for the survival and differentiation of dopaminergic and spinal cord neurons and for certain subpopulations of hippocampal neurons. In the CNS, the highest levels of BDNF mRNA are found in the hippocampus. As seen for other factors, BDNF level is rapidly increased in brain after various types of gliosis-promoting damages induced by physical lesion, spinal cord crushing, kainic injection, seizures or ischemia (31). Several authors have reported modulation of the expression of a truncated form of the BDNF receptor TrkB after injury (32). Expression of this truncated form, which lacks the catalytic tyrosine kinase (TK) domain, increases and stays elevated for at least 2 mo. This suggests that BDNF and TrkBTK- play a role in dopaminergic regeneration and repair. The truncated TrkB which is expressed in reactive astrocytes may act as negative regulators of neurite growth in damaged regions (33). After injury, BDNF can prevent neuronal loss and promote some regeneration and this is also observed in vitro. Transplants of fibroblasts genetically modified to express BDNF promote regeneration of adult rat rubrospinal and spinal cord after injury in the adult rat.

3.6.3. Neurotrophin-3 (NT-3)

NT-3 is a neurotrophic factor which prevents the death of adult central noradrenergic neurons in vivo, but it also induces microglia proliferation and phagocytic activity, sug-

gesting that the factor plays a role in cellular activation. After crushing of the spinal cord, an increased level of BDNF and NT3 mRNAs is first observed at 6 h and the labeling is enhanced at 24 h and 72 h (34). This NT-3 promotes growth of lesioned dorsal column axons with an abundance of fiber sprouting apparent at the lesion site, and many fibers extending into and beyond the lesion epicenter. After brain injury, a moderate increase in BDNF mRNA expression is observed bilaterally in the CA3 region of the hippocampus at 1, 3, and 6 h, but expression declined to control levels by 24 h. Conversely, NT-3 mRNA is significantly decreased in the dentate gyrus (35). Topical application of NT-3 attenuates ischemic brain injury after transient middle cerebral artery occlusion in rats.

3.6.4. *Glia Cell Line-Derived Neurotrophic Factor (GDNF)*

After a wire knife in adult mouse striatum GDNF mRNA expression increases within 6 h, doubles after 1 wk and remains elevated for at least 1 mo. Most GDNF expression is associated with brain macrophages. The dopaminergic sprouting that accompanies striatal injury appears to result from neurotrophic factor secretion by activated microglia at the wound site. An increase in GDNF receptors occurs in neurons after ischemia or facial nerve injury (36). The main known effect of GDNF is to protect nigral dopaminergic neurons involved in Parkinson's disease. GDNF also protects motoneurons and calbindin neurons. In addition, the increase in nitric oxide that accompanies ischemia and subsequent reperfusion is blocked almost completely by this protein. Thus, GDNF may be efficient in the treatment of cerebrovascular occlusive disease.

3.7. *Cytokines (Interleukins, Tumor Necrosis Factor- α , Interferon- γ)*

Traumatic injury to the CNS initiates inflammatory processes that are implicated in secondary tissue damage. These processes include the synthesis of pro- and anti-inflammatory cytokines, leukocyte extravasation, vasogenic edema, and blood-brain-barrier breakdown. IL-1, IL-6, TNF and IL-8, which are increased after head injury, play a role in the cellular cascade of injury (37). Many cytokines (IL-1, IL-2, IL-6, TNF- α and IFN- γ) induce a significant increase of astrogliosis.

3.7.1. *Interleukin-1 (IL-1)*

IL-1 β is a proinflammatory cytokine, produced by blood-borne and resident brain inflammatory cells. IL-1 level is regulated through the activity of the specific protease IL-1 β converting enzyme. This enzyme dependence is suggested by its ischemia-induction and by the reduced brain reaction when it is inhibited. IL-1 is produced by astrocytes (38), by microglia (39), by some neurons and by endothelial cells. IL-1 β is synthesized early after ischemia or after other brain damages and may play a role in postlesion recovery. After injury, IL-1 receptors are located on neurons and, at the site of gliosis, on astrocytes and microglia (37a).

IL-1 is a potent mitogen for astrocytes in which it stimulates the production of cytokines such as NGF, PDGF, TNF- α and IL-6, which, in turn, will act on neurons and on microglial cells. Increase of IL-1 reduces ischemic and excitotoxic brain, however, deleterious effects have also been reported like exacerbation of ischemic brain damage, and blocking of IL-1 activity can be beneficial for ischemic brain edema. Thus, excessive production of IL-1 appears to mediate experimentally induced neu-

rodegeneration in vivo, whereas neuroprotective effects of low concentrations of the cytokine suggest a dual role for IL-1 in neuronal survival (40). An endogenous IL-1 receptor antagonist (IL-1ra) prevents binding of IL-1 β to the signaling receptor. It was found to attenuate or inhibit neuronal damage in several types of injury. IL-1ra also suppresses NGF production in injured rat brain. The fact that IL-1ra inhibits neuronal damage in the rat confirms that endogenous IL-1 is a mediator of this type of damage. Thus, inhibitors of IL-1 action may be of therapeutic value in the treatment of acute or chronic neuronal death.

3.7.2. Interleukin-2 (IL-2)

IL-2 is an immunoregulatory cytokine, initially discovered for its mitogenic activity on T cells. It also acts on monocytes, resulting in the activation of cytokine production, superoxide production, and tumoricidal activity. IL-2 is present in injured rat brain, mainly in the lesion site, reaching a maximal activity at 10 d postlesion. LPS- or IL-2-activated microglia express IL-2 receptor beta-chain protein in culture (41). IL-2 treatment results in an intracranial accumulation of T and B lymphocytes within the infused brain hemisphere. Adjacent brain regions were characterized by reactive astrogliosis, microglial activation, endothelial upregulation of adhesion molecules, myelin damage and neuronal loss. Severe brain damage is observed in IL-2-transgenic mice but IL-2 elicits some neurotrophic effects (42).

3.7.3. Interleukin-3, -4, and -5 (IL-3, IL-4, IL-5)

IL-3 (multi-CSF) is a hematopoietic and immunomodulatory cytokine. It stimulates the proliferation and activation of microglia and can enhance differentiation of cholinergic and sensory neurons. Chronic CNS production of low levels of IL-3 promotes the recruitment, proliferation and activation of macrophage/microglial cells in white matter regions with consequent primary demyelination and motor disease (43). Microglia produce IL-3 constitutively with the probable activation of an autocrine loop. Astrocytes too, synthesize IL-3. This factor prevents delayed neuronal death in the hippocampal CA1 field.

IL-4 is a cytokine which plays an important role in the function of various immunocompetent cells as well as in the pathophysiology of various CNS disorders. It inhibits astrocyte activation and induces NGF secretion (44). IL-4 induces proliferation and activation of microglia.

IL-5 is a microglia mitogen which is secreted by both microglia and astrocytes in response to inflammatory stimuli. Thus, IL-5 may be involved in the cytokine-immune cascades leading to microglia proliferation in damaged areas.

3.7.4. Interleukin-6 (IL-6)

IL-6 is a proinflammatory cytokine with neuroprotective properties. In the intact brain, IL-6 is mainly localized in cholinergic and GABAergic neurons of the basal forebrain. After brain damage, there is a rapid and concomitant increased expression of IL-1 β (45), IL-6 and their receptors (46). Their predominantly neuronal localization in rat brain suggests a role for IL-6 in activating micro- and astroglial cells in response to degenerating cholinergic neurons. However, it has been reported that after traumatic injury, IL-1 and TNF- α induce IL-6 production by astrocytes (47). IL-6 is mitogenic for astrocytes and it induces NGF production in these cells, suggesting that

the IL-6 producing brain injury may likely contribute to the release of neurotrophic factors by astrocytes. Overexpression of IL-6 in astrocytes or in neurons (IL-6 transgenic mice) (48), or of IL-6/soluble IL-6R alpha (49), induce massive reactive astrogliosis and microgliosis (48). Accelerated regeneration of the axotomized nerve observed in these transgenic mice suggests that the IL-6 signal may play an important role in nerve regeneration after trauma. In IL-6 knock-out mice, the initial inflammatory response to cortical injury is diminished (50). These results emphasize the role of IL-6 in the global regulation of neurons, astrocytes, and microglia and their activation in the injured nervous system.

3.7.5. Interleukin-8, -12 (IL-8, IL-12)

IL-8, a neutrophil-activating cytokine that promotes invasion of these leukocytes into brain parenchyma, is not expressed in normal brains. It is expressed after reperfusion in brain ischemia and contributes to edema. IL-8 can be induced in astrocytes surrounding tumor lesion (51), after acidosis, and in microglia, after LPS treatment (52). IL-12, a proinflammatory cytokine which activates cellular immune response mechanisms, has a significantly elevated level in all patients in the course of 14 d after severe head trauma (53). The upregulation of IL-12 in activated microglia depends on an autocrine loop of TNF- α .

3.7.6. Interleukin-10 (IL-10)

IL-10, a cytokine with antiinflammatory properties, negatively modulates proinflammatory cascades at multiple levels. It is a potent inhibitor of cytokine synthesis by microglia. After traumas, IL-10 can be found in the CNS and in the CSF (54). Rapid monocytic IL-10 release after sympathetic activation may represent a common pathway for immunodepression induced by stress and injury. Acute stroke significantly increases CSF levels of IL-8 and IL-10 (55). IL-10 and its receptor mRNA are enhanced in both microglia and astrocytes after stimulation of these cells with LPS. After traumatic brain injury, IL-10 treatment improves neurological recovery and significantly reduces TNF expression. This improvement may relate, in part, to reductions in proinflammatory cytokine synthesis like TNF- α , IL-12, and NO production by microglia (56). Four days after local application of IL-10 to the site of corticectomy in adult mice, the number of reactive astrocytes and their state of hypertrophy were reduced (by 60%). In control mice, IL-10 induced a dose-dependent increase of NGF secretion in astrocytes.

3.7.7. TNF- α

The cytokine tumor necrosis factor (TNF- α) is a pleiotrophic polypeptide that plays a significant role in brain immune and inflammatory activities. TNF- α is produced in the brain in response to various pathological processes such as infectious agents, ischemia, and trauma (37). TNF- α is present early in neuronal cells, in and around the ischemic tissue, and later in macrophages and in astrocytes (57). Exposure of glial cells to the cerebrospinal fluid of AD patients was followed by TNF- α release (58). TNF- α activates glial cells, thereby regulating tissue remodeling, gliosis, and scar formation. IL-1 β and TNF- α synergistically stimulate NGF release from cultured rat astrocytes. They downregulate the expression of GFAP. TNF- α , IL-1 and, to a lesser degree, IL-6, are mitogenic for astrocytes in vitro. However, TNF- α inhibits prolifera-

tion induced by other mitogens. Thus, TNF- α may be important in regulating the proliferative response of astrocytes during reactive astrogliosis in vivo (59). Tumor necrosis factors protect neurons against metabolic-excitotoxic insults and promote maintenance of calcium homeostasis.

3.7.8. Interferon-gamma (IFN- γ)

IFN- γ , appear to have complex and broad-ranging actions in the CNS that may result in protection or injury. It is a potent microglia/macrophage activator. Although IFN- γ is induced after injury, it does not appear as prerequisite for gliosis induction since in IFN- γ knockout adult mice, astrogliosis and increases of IL-1 α and TNF- α levels are induced rapidly by injury (60). However, IFN- γ can induce gliosis and CNS demyelination (61). IFN- γ specific transcripts are detected in rat astrocytes and are upregulated after treatment with IFN- γ itself. IFN- γ increases synthesis of NGF in reactive astrocytes but not in the normal adult astrocytes. It has been reported to inhibit the proliferation of rat cortical astrocytes and to reduce the levels of the ECM molecules. The production of IFN- γ in the CNS can be a two-edged sword that on the one hand confers protection against a lethal viral infection but on the other hand causes significant gliosis to the brain.

3.8. Leukemia Inhibitory Factor (LIF) and Oncostatin M (OSM)

LIF mRNA is increased 30-fold 6 h after surgical lesion of the cortex in adult rat brain, it reaches a peak at 24 h and returns to baseline after 7 d. LIF mRNA is induced in GFAP-positive astrocytes as well as in a small number of microglial cells. The striking induction of LIF transcripts in glia suggests that this cytokine may play a key injury-response role in the CNS as it does in the PNS, where LIF has been demonstrated to regulate neuropeptide expression both in vivo and in vitro (62). In LIF knock-out mice, the microglial and astroglial responses to surgical injury of the cortex are slower compared to wild-type mice. LIF is a key regulator of neural injury in vivo, where it is produced by glia and can act directly on neurons, glia, and inflammatory cell (50). Levels of OSM, LIF, and CNTF were all increased in the hippocampus after seizure, to different extents and with different time courses. The majority of LIF+ cells were astrocytes, while the majority of OSM+ cells had the morphology of interneurons (63).

3.9. Ciliary Neurotrophic Factor (CNTF)

CNTF elicits trophic effects on ciliary, motor sympathetic, sensory, retinal and hippocampal neurons. It ameliorates axotomy-induced degeneration of CNS neurons and is upregulated at wound sites in the brain (64). The increased level of CNTF mRNA in lesioned hippocampus is maximal by 3 d and is sustained for up to 20 d. Increased CNTF level in CNS recapitulates the glial response to CNS lesion with astrogliosis and appearance of activated microglia (65). These data are consistent with a model whereby CNTF (which is synthesized and stored by astrocytes) would be released when the integrity of the astrocyte membrane is compromised, whereupon it would elicit an inflammatory response. CNTF has neurotrophic and neuroprotective effects, but more limited than those of other neurotrophic factors. It prevents medial septum neuron degeneration and promotes low affinity NGF receptor expression in the adult

rat CNS. CNTF stimulates astroglial hypertrophy in vivo and in vitro and GFAP expression (66,67).

3.10. Hepatocyte Growth Factor (HGF/SF)

In brain, HGF/SF is found in astrocytes and microglia, as well as in rare cortical neurons. Expression of both HGF and c-Met/HGF receptor was markedly induced in response to cerebral ischemic injury (68). HGF prolonged survival of embryonic hippocampal neurons in primary culture. In vivo, it has a profound neuroprotective effect against postischemic delayed neuronal death in the hippocampus.

3.11. Thrombin

Thrombin is a protease/growth factor the effects of which are mediated by the protease activated receptors (PAR-1, 2, 3). This factor is liberated in the site of injury by the blood clotting process but it is also synthesized by nerve cells (69). Its involvement in gliosis is suggested by various observations. Infusion in rat caudate nucleus caused infiltration of inflammatory cells, proliferation of mesenchymal cells, induction of angiogenesis, and an increase in vimentin-positive reactive astrocytes (70). Thrombin inhibitors ameliorate secondary damage in rat brain injury by suppressing inflammatory cells and vimentin-positive astrocytes (71,72). Thrombin activates cultured rodent microglia (73) and activates intracellular death protease pathways inducing apoptosis in model motor neurons (74). It is an endogenous mediator of hippocampal neuroprotection against ischemia at low concentrations but causes degeneration at high concentrations (75).

4. GROWTH FACTORS AND GLIOSIS IN THE AGING BRAIN

4.1. Alterations Linked to Naturally Occurring Gliosis

In aged rats, the volume of hippocampus was higher while that of frontal cortex was unchanged. Various brain tissue alterations have been detected by the use of magnetic resonance imaging (MRI). With advancing age, the periventricular and subcortical white matter becomes susceptible to a heterogeneous assortment of such alterations that cannot be easily categorized in terms of traditionally defined neuropathologic disease. Frequently observed microscopic changes include dilated perivascular (Virchow-Robin) spaces, mild demyelination, gliosis, and diffuse regions of neuropil vacuolation. Associated clinical abnormalities are usually confined to deficits of attention, mental processing speed, and psychomotor control. Occasionally, histologically severe white matter lesions may occur that result in dementia and focal neurologic impairment. These lesions are characterized by extensive arteriosclerosis, diffuse white matter necrosis, and lacunar infarction (76). Some results suggest that in aging, gliosis is secondary to the neurodegenerative changes. Synaptic loss is likely to be a common pathogenetic feature of aging and neurodegenerative disorders and a likely cause of clinical symptoms (77).

4.2. Microglia

With aging, microglial cells grow more numerous, become activated and may enter the phagocytic or reactive stage. They also become richer in iron and ferritin and exhibit phenotypic alteration, e. g., the expression of MHC class II antigens that are

not ordinarily demonstrable immunohistochemically in the resting state (78) and an increase in TGF- β 1 level. Microglial cells are prominently involved in such pathologic processes as the acquired immunodeficiency syndrome, multiple sclerosis, prion diseases, and the degenerative disorders, e.g., Alzheimer's disease and Parkinson's disease.

4.3. Astrocytes

The aging process increases astrocyte and pericyte populations in the rat parietal cortex (79). The predominant change is glial activation characterized first by GFAP expression. In human, GFAP level increases dramatically after the age of 65 yr, and more especially in the hippocampal formation. This glial reaction was observed in aged patients that do not show cognitive impairment and the neuropathological hallmarks of Alzheimer's disease (80). GFAP is a unique example of a gene that shows increased expression during aging. Astrocytosis and production of APP-derived fragments occur markedly in senescence-accelerated mice (SAMP-8) brains. During aging, astrocytes also exhibit signs of metabolic activation, an hypertrophy of their cell body and processes and the extension of these astrocytic processes around neurons. Tissue levels of S100 β as well as the number of S100 β -immunoreactive astrocytes, increased with advancing age. In hippocampus, astrocytes lose their radial organization.

Age has a differential effect on astrocytic and microglial hyperactivity in gray vs white matter areas. This mosaic of glial aging suggests that multiple mechanisms are at work during aging (81). The regional heterogeneity of aging changes has been reported by many authors. For example, glial expression of GFAP, apolipoprotein E, apolipoprotein J (clusterin), heme oxygenase-1, complement 3 receptor (OX42), MHC class II and TGF- β 1 were elevated in the corpus callosum during aging but other regions showed marked dissociation of the extent and direction of changes (81). The change in glial volume and ECM observed in old animals, results in a decrease in the apparent diffusion coefficient of neuroactive substances. This could lead to the impairment of the diffusion of neuroactive substances, extrasynaptic transmission, "crosstalk" between synapses and neuron-glia communication.

4.4. Origin of Age-Related Gliosis

The origin of age-related gliosis is not known but since gliosis is supposed to be triggered by neural lesions, the presence of such lesions in patients over 60 yr has been investigated by using MRI and microscopic observation (82). Periventricular lesions graded as moderate or severe were found in 10% of the patients in the age group between 60 and 69 yr, and in 50% between 80 and 89 yr. The presence or absence of periventricular lesions on MRI correlated well with the severity of demyelination and astrocytic gliosis. Demyelination was always associated with an increased ratio between wall thickness and external diameter of arterioles (up to 150 microns). A variable degree of axonal loss in Bodian-stained sections was present in the white matter of all brains with demyelination. Dilated perivascular spaces were found; their presence correlated strongly with corrected brain weight, but incompletely with demyelination and arteriolosclerosis. The observations suggest that arteriolosclerosis is the primary factor in the pathogenesis of diffuse white matter lesions in the elderly. This is soon followed by demyelination and loss of axons, and only later by dilatation of perivascu-

lar spaces (82). Some other histological changes appear to be universal in aged human brains. These include increasing numbers of corpora amylacea within astrocytic processes near blood-brain or cerebrospinal fluid-brain interfaces, accumulation of the "aging" pigment lipofuscin in all brain regions, and appearance of neurofibrillary tangles (but not necessarily amyloid plaques) in mesial temporal structures.

An increasing body of evidence indicates that aging-related impairments of nervous functions are caused by loss of neurons. It has been found that about half of the motoneuron somata examined by electron microscopy, from aged Sprague-Dawley rats, had a reduced (50%) bouton coverage, which seemed to be caused by a smaller number of axosomatic bouton profiles. Long stretches of the cell body plasma membrane were apposed by pale processes, and immunolabeling for GFAP disclosed that a number of the aged motoneurons appeared embedded in GFAP immunopositive processes. Astrogliosis is also observed in the spinal cord of old rats (83).

Two mutant mice have been compared, SAM-P8 characterized as mice in which aging is accelerated with occurrence of memory disturbances and the senescence-resistant (SAM-R1) mice. The concentrations of the β -subunit of NGF in the hippocampus were reduced at 8-mo-of-age in both mutants but the decrease was more conspicuous in SAM-R1. In the olfactory bulbs, the β -NGF level was already higher in SAM-P8 than in SAM-R1, at 2-mo-of-age. The acceleration of age-related increase was apparent in the levels of S100 β and Mn-SOD in the cerebral cortex from SAM-P8. In these last mutants, a fibrous gliosis was present at quite an early age in selected regions of the brain (84). In contrast, in aged rats, there is a reduced content of NGF in the frontal and parietal cortices. Repeated oral administration of the stimulators of the NGF synthesis, idebenone and propentofylline, produced a significant recovery of the reduced NGF content (85).

4.5. Inhibition of Gliosis

In enriched environment conditions, there are less astrocytes and they are less hypertrophied than in naive animals, GFAP level is also lowered (86). In general, the effects of steroids oppose the effects of aging. Recent data indicate that steroid treatment can decrease the expression of GFAP in the aged brain, yet GFAP is resistant to downregulation by endogenous glucocorticoids (87).

4.6. Gliosis and Release of Trophic Factors after Brain Lesion in the Aging Rat

To our knowledge there are few data about gliosis following damage in aged animals. As seen before, diffuse glial reaction occurs naturally in aging brain, but most studies deal with gliosis which is associated to age-related diseases or which is artificially induced.

4.6.1. Lesions Induced in Normal Animals

In the deafferented neostriatum produced by unilateral 6-hydroxydopamine injection in 6-, 15-, or 24-mo-old-rats, the time-dependent induction of GFAP was larger and persisted longer in the aged rats. The response of middle-aged rats was intermediate. After stab wounds in the neostriatum, there were substantially larger GFAP inductions than after deafferentation, but fewer effects of age. In both lesion paradigms, GFAP staining increased in the contralateral striatum of old rats, but not in young rats.

The consistency of the exaggerated glial reactivity in the aged brain after modest injury involving either the nigrostriatal system or the septohippocampal one suggests some remarks. It appears that aged astrocytes are more sensitive to gliotrophic factors released by terminal degeneration. Larger quantities of such factors are produced after injury and their clearance is delayed in old rodents and/or aged astrocytes are less able to terminate GFAP inductions after activation (88).

The Cotman group performed deafferentation of the hippocampus by ablation of the entorhinal/occipital cortex in young and aged rats. These last rats in which reactive sprouting is deficient showed no increase in neurite-promoting activity by 12 d after hippocampal deafferentation while young animals had a several fold increase of this activity between 9 and 15 d postlesion (89). In middle-aged and aged-rats, deafferentation of dentate gyrus induces reactive axonal growth by surviving afferent systems, which is delayed and reduced relative to young adults (90). The cause for this age-related decline may reflect a decrement in trophic signals which initiate sprouting. IGF-1 may play a role in sprouting because it promotes axonal growth and it is expressed at elevated levels by microglia just prior to sprouting onset. It is also expressed with better spatiotemporal correspondence to hippocampal sprouting than other trophic factors. Messenger RNA levels of IGF-1 were markedly elevated 4 d after an entorhinal cortex lesion performed at 3-mo-of-age and there was only a modest increase 8 d after the lesion performed at 18–26-mo-of-age. These results support the association between IGF-1 expression impairment in microglia and reduced axonal plasticity with age.

Following a unilateral ibotenic acid lesion of the nucleus magnocellularis (NBM) in 3- and 15-mo-old rats, there was a loss of NBM choline acetyltransferase-(ChAT)-positive cells, a decrease in cortical ChAT activity and impairment of the acquisition of avoidance response. When NGF was administered with GM1 ganglioside immediately after surgery, there was an attenuation of the lesion-induced changes, however, GM1 had no effect on ChAT activity decrease and behavioral impairment in 15-mo-old rats. The age effect on gliosis resulting from brain lesion has characteristics which depend on the type of lesion (mechanical or chemical) and on the cerebral structure concerned. After total immunolesion resulting from intraventricular injections of the immunotoxin, 192 IgG-saporin in aged rats, there was an increase of microglia but not astrocytes in the subnuclei of basal forebrain. This immunotoxin was effective in young and aged rats, in producing cholinergic lesions. NGF protein levels were significantly increased in the hippocampus, cortex, and olfactory bulb of the young rats but not of the aged rats, except for small increases in the olfactory bulb. The total immunolesions had no effects on NGF and BDNF mRNA levels in the hippocampus and cortex (91). No difference was found between normal young adult (2–5 mo) and aged (24 mo) rats in terms of hippocampal NGF levels. However, following medial septal lesions, only young rats demonstrated significant increases in hippocampal NGF-like activity (92).

4.6.2. Lesions Occurring in Transgenic Animals

Homozygous beta amyloid precursor protein (APP)-deficient mice are viable and fertile. However, the mutant animals weighed 15%–20% less than age-matched wild-type controls. Neurological evaluation showed that the APP-deficient mice exhibited a decreased locomotor activity and forelimb grip strength, indicating a compromised

neuronal or muscular function. In addition, four out of six homozygous mice showed reactive gliosis at 14-wk-of-age (93). These null mice also had marked reactive gliosis in many areas, especially in cortex and hippocampus throughout the CA1 region (11). It is not known if the impaired synaptic plasticity and long term potentiation are related to gliosis. Normal β -APP may serve an essential role in the maintenance of synaptic function during ageing. Alteration of this function may contribute to the progression of the memory decline and the neurodegenerative changes seen in Alzheimer's disease. Elevated GFAP levels have been reported in transgenic mice expressing the gene for bovine growth hormone (bGH) with increased body size, reduced reproductive capacity, and high basal levels of several hormones including corticosterone (94). This may reflect increased neural damage due to accelerated aging processes or damage associated with high circulating levels of bGH or corticosterone.

Alternatively, this increased expression of GFAP may reflect altered regulation of GFAP rather than an increased signal by neural damage. Young glycosyl asparaginase-deficient mice demonstrate many pathological changes found in human aspartylglycosaminuria patients. After the age of 10 mo, the general condition of null mutant mice gradually deteriorate, with a progressive motoric impairment and impaired bladder function until premature death. A widespread lysosomal hypertrophy in CNS was detected. The oldest animals (20-mo-old) display a clear neuronal loss and gliosis, particularly in brain regions with the most severe vacuolation. The severe ataxic gait of the older mice is likely due to the dramatic loss of Purkinje cells, intensive astrogliosis, and vacuolation of neurons in the deep cerebellar nuclei, and the severe vacuolation of the cells in vestibular and cochlear nuclei (95).

4.6.3. Administration of Growth Factors

There is a reduced content of NGF in the frontal and parietal cortices of aged rats. Repeated oral administration of the stimulators of NGF synthesis, idebenone, and propentofylline, produced a significant recovery of the reduced NGF content (85). With trimethylquinone derivative, they induced an increase in NGF protein and mRNA, and in choline acetyltransferase activity, in basal forebrain-lesioned and aged rats, but not in intact young rats. Many authors reported the reparative effects of NGF with survival of the cholinergic neurons and regrowth of damaged fibers after fimbria fornix lesion in aged rats. Atrophic cholinergic neurons in aged animals were similarly responsive to NGF treatment, like those in the young animals. The responses of basal forebrain cholinergic neurons to NGF treatment varied with time after the lesion and imply that NGF administration could promote the collateral sprouting from spared cholinergic fibers after the lesion in the septohippocampal system and forebrain. A role for glial cells was proposed to clarify how NGF availability may be regulated during the degenerative and regenerative events. Concerning the neostriatum, it has been reported that neostriatal NGF binding sites and intrinsic cholinergic neurons are co-localized and are lost to an equal extent following age- or injury-induced loss of neostriatal neurons (95A). It appears that NGF has potential use as a cholinergic "neurotrophic-factor therapy." Stimulation of glucocorticoid or beta-adrenergic receptors by dexamethasone and clenbuterol, respectively, has been shown to increase NGF mRNA levels in adult rat brain. A more modest increase was observed for the aged rats. The variation throughout life, of induction of NGF expression by neurotransmitter/hormone receptor activation

suggests that pharmacological agents might be useful tools to enhance trophic support in aging. Concerning the NGF-receptor, a reduced immunoreactivity was found within the nucleus basalis of Meynert (NBM) neurons in aging (18–24 mo) rats (96).

4.7. Growth Factors in Gliosis Resulting from Age-Related Diseases

Aging is accompanied by increases in glial cell activation, in oxidative damage to proteins and lipids, in irreversible protein glycation, and in damage to DNA, and such changes may underlie in part the age-associated increasing incidence of degenerative conditions such as Alzheimer's disease (AD) and Parkinson's disease. Then the degenerative diseases can induce gliosis. Aging is also characterized by an accumulation of lipofuscin pigments, formation of paired filaments and increased immunoreactivity to microtubule-assembling protein tau occurring in paired filaments of neurofibrillary tangles, beta-amyloid proteins, and ubiquitin (97).

Astrogliosis is observed in AD (98) and it appears that the neurons and their processes although not exclusively may be the site of EGF-R immunoreactivity. An EGF/EGF-R system within the CNS may play an important part in scar formation in response to neuronal injury and death occurring in AD and also in aging. It may also function as a trophic factor important in axonal or dendritic sprouting. FGF-2 is increased in the brains of AD patients. Furthermore, FGF-2 is not distributed in an identical fashion to normal and AD brains, but is found in association with the lesions that characterize this disease. PDGF is associated with neuronal and glial alterations of AD. Antibodies against PDGF-BB (but not PDGF-AA) recognized the neurofibrillary alterations of this disease. The levels of PDGF-BB correlated with the patterns of synaptic loss and sprouting while PDGF-AA immunostaining of the vessels was correlated with glial proliferation. PDGF protein was mildly increased in AD. These data suggest that PDGF, as well as other neurotrophic factors, play an important role in the mechanisms of neurofibrillary pathology. TGF- β 1 is in senile plaques in AD and limited to neuritic profiles within them. TGF- β 2 is seen in neuronal neurofibrillary tangles, plaque neurites, microglia, astrocytes and macrophages. TGF- β 3 is restricted to Hirano bodies. Such expression is not observed in every gliosis since in infarction, reactive astrocytes and macrophages express the three isoforms. The localization of TGF- β isotypes to the lesions of AD supports the hypothesis that these cytokines may influence lesion expression. Overexpression of TGF- β 1 may initiate or promote amyloidogenesis in AD (99) and could be related to its capacity to induce ECM component production which could promote the formation of amyloid plaques (100). It has been shown that TGF- β can increase amyloid-beta protein immunoreactive plaque-like deposits in rat brain. This effect could be exerted by astrocytes in which TGF- β 1 elevates APP mRNA level, and also increases the half-life of APP message by at least five-fold (101). Finally, induction of TGF- β 2 which occurs in various degenerative diseases, may be an intrinsic part of the processes that induce neurofibrillar tangles formation and reactive gliosis.

Gliosis is also detected in vascular dementia. In Creutzfeldt-Jakob disease, there is a diffuse astrogliosis determined by cell hypertrophy (102) and elevation of GFAP in the later stages of disease. Microgliosis is also observed in the grey matter where astrogliosis and prion protein (PrP) deposits were prominent. Activated microglia may contribute to neuronal damage due to their cytotoxic potential (103). In areas where

spongiform degeneration affected the entire depth of the cortex, microglia exhibited atypical, tortuous cell processes, and occasionally intracytoplasmic vacuoles, suggesting that microglia themselves may become a disease target.

Following bacterial or viral infections, toxic products such as bacterial LPS circulate to the brain, where they induce IL-1 and iNOS mRNA synthesis. After several hours delay, massive quantities of NO are released. The large amounts of NO released by iNOS may well produce death of not only neurons but also glial cells. NO stimulates glutamic acid release, which by N-methyl-D- aspartate (NMDA) receptors causes release of NO leading to neuronal cell death. Repeated bouts of systemic infection could result in induction of iNOS in the CNS and lead to large fall out of neurons in hippocampus to impair memory, in hypothalamus to decrease fever, and neuroendocrine response to infection, and could play a role in the pathogenesis of degenerative neuronal diseases of aging, such as AD. The damage to the pituitary could also impair responses to stress and infection, and the release of NO during infection could be responsible for the degenerative changes in the pineal and diminished release of melatonin, an antioxidant, and consequently, an antiaging hormone, that occur with age(104).

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Role of Fibroblast Growth Factor-2 in Astrogliosis

John F. Reilly

1. INTRODUCTION

Astrocytes comprise the most numerous cellular element of the brain. Though once thought to merely provide structural support for neurons, astrocytes are now known to participate in many aspects of normal nervous system development and function, including neuronal migration, synaptogenesis, induction of the blood-brain-barrier, metabolic support of neurons, and regulation of extracellular fluid composition. Astrocytes throughout the brain are electrically coupled, forming a glial syncytium that may play a substantial role in signaling within the nervous system (1). In addition to functions in the developing and adult nervous systems, astrocytes respond to changes in the brain caused by aging and injury.

2. ASTROGLIOSIS

The responses of astrocytes to aging and injury are termed astrogliosis, and can include proliferation, an increase in cell number through mitosis, and hypertrophy, an increase in cell size and process outgrowth. Many ultrastructural changes accompany astrogliosis, including enlargement of the nucleus, increased numbers of mitochondria and lysosomes, and increased glycogen. A diverse array of molecules, including various cell adhesion proteins, major histocompatibility complex class I and II proteins, early-response gene products, cytokines and growth factors, proteases, and protease inhibitors are upregulated in astrocytes following injury (2). However, the most striking ultrastructural feature of astrocyte hypertrophy is the increased cytoplasmic accumulation of glial fibrillary acidic protein (GFAP). GFAP is the principal intermediate filament of the astrocyte cytoskeleton (3), and immunostaining for GFAP is commonly used both to identify astrocytes and to assess their morphological changes.

2.1. Age-Related Astrogliosis

Ultrastructural studies have demonstrated hypertrophy of astrocytes in the aging brain (4). The increases in astrocyte cell size with advancing age are not accompanied by an overall increase in the number of astrocytes identified by morphological criteria, suggesting that age-related astrogliosis is characterized primarily by hypertrophy, rather than proliferation. GFAP protein levels have also been shown to increase with

age in the mouse, rat, and human brain (5–8). This increase occurs in both gray and white matter in several brain regions. Though an increase in the number of GFAP-positive astrocytes has been observed with aging (9,10), this most likely results from enhanced immunodetection due to increased GFAP expression. Astrocytes in the intact central nervous system (CNS), especially protoplasmic astrocytes in the gray matter, normally express very low levels of GFAP protein, which can be below the threshold for routine immunohistochemical stains (11).

The increases in GFAP protein content result from an increase in the amount of GFAP mRNA in astrocytes (6,12,13). As with the increase in GFAP protein expression, this increase occurs in many brain regions, and progresses with advancing age. However, the increased expression of GFAP mRNA does not appear to result from increased transcription (14). This suggests that age-induced astrogliosis is associated with increased stability of GFAP mRNA, protein, or both.

2.2. Injury-Induced Astrogliosis

Astrocytes exhibit one of the earliest and most remarkable cellular responses following a variety of insults to the CNS. Both proliferation and hypertrophy of astrocytes occur, and it is likely that these events are independently regulated. It has been shown that in response to facial nerve transection, astrocytes in the rat facial nucleus exhibit hypertrophy in the absence of an appreciable proliferative response (15,16). This phenomenon has also been demonstrated in the rat visual cortex following optic nerve transection (17). Following a traumatic injury to the CNS, only a small proportion of astrocytes undergo cell division, suggesting that astrocyte hypertrophy is the major determinant of trauma-induced astrogliosis (18–20).

Very few studies of astrocyte hypertrophy have relied on morphological criteria; in general, astrocyte hypertrophy is evaluated using GFAP immunostaining. Astrocyte hypertrophy and the concomitant increase in expression of GFAP mRNA and protein are detectable less than 24 h after a traumatic injury (21–23). The response is likely to be more rapid than these data suggest, since expression of a reporter gene driven by the GFAP promoter is detectable as early as one hour following traumatic injury in a transgenic mouse model (24). Expression of GFAP mRNA and protein reach a peak between seven and 14 d following injury, and persist at elevated levels beyond 30 d. Although the time course varies with the nature and severity of the injury, expression of GFAP is increased in a wide variety of experimental models, including ischemia, systemic administration of kainic acid, and deafferentation.

Astrocytes in the aged brain respond differently to injury than those in the neonatal or adult brain. In deafferentation models of injury, astrocytes in the aged brain demonstrate increases in GFAP mRNA and protein that are greater in magnitude than in adult rats (25,26). These increases appear to develop more slowly in the aged rodent brain, and persist longer. The delayed but stronger response to injury may reflect a difference in the astrocytes of the aged brain, or may result from alterations in the factors that regulate astrogliosis.

3. FIBROBLAST GROWTH FACTOR-2

The response of astrocytes to injury, including alterations in the expression of GFAP, is thought to be initiated and regulated by local chemical mediators released at the site

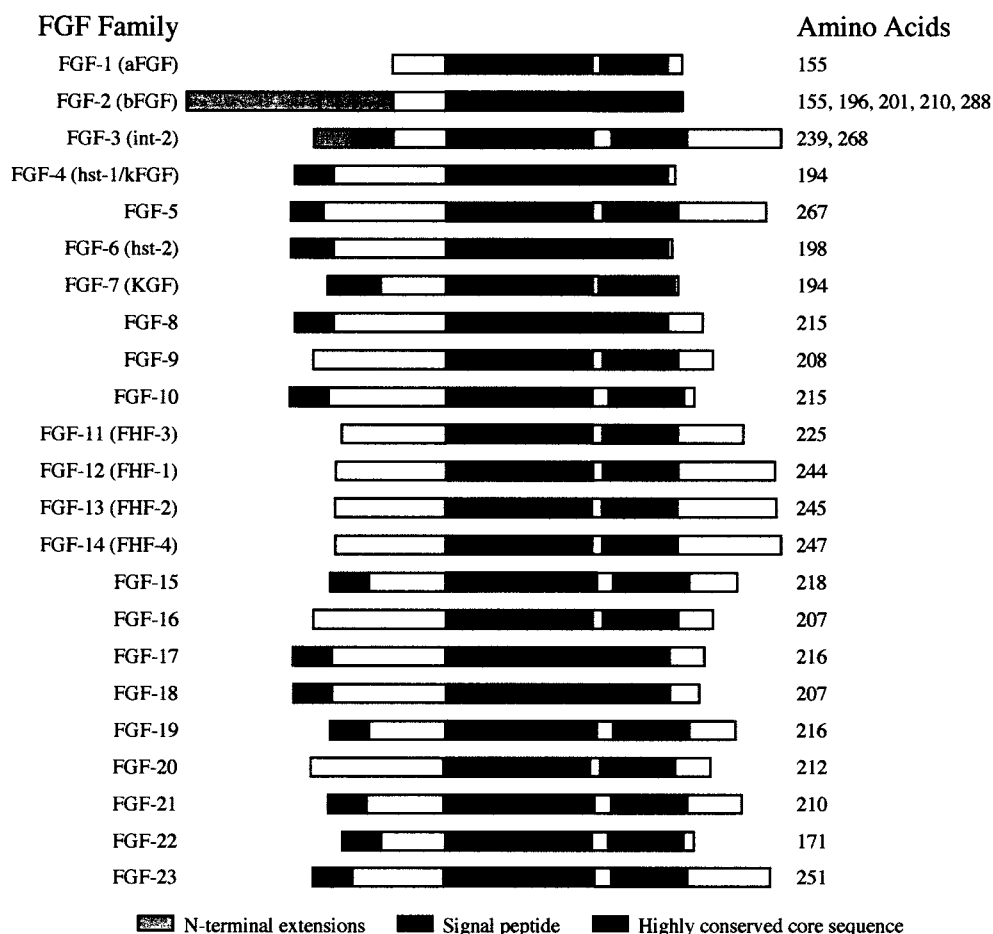


Fig. 1. Structural features of the FGF family. The core structure contains the regions of greatest sequence identity between family members. A hydrophobic signal peptide is present on several of the FGFs. N-terminal extensions of FGF-2 and -3 represent alternate initiation sites, and contain nuclear localization sequences. References: FGF-1 (29–31), FGF-2 (28,34,35), FGF-3 (33,133), FGF-4 (134,135), FGF-5 (136), FGF-6 (137), FGF-7 (138), FGF-8 (139), FGF-9 (46), FGF-10 (140), FGF-11 and -12 (141), FGF-13 and -14 (142), FGF-15 (143), FGF-16 (45), FGF-17 (144,145), FGF-18 (146,147), FGF-19 (148), FGF-20 (47), FGF-21 (149), FGF-22 (GenBank AB021925), FGF-23 (150).

of injury. Many growth factors and cytokines have been identified in the wounded rat brain, and some or all may be involved in the activation of glial cells. Substantial evidence suggests that basic fibroblast growth factor (FGF-2) plays a significant role in the response of astrocytes to injury.

3.1. FGF Gene Family

The FGF family comprises a rapidly expanding group of polypeptide growth factors with 23 members identified to date (Fig. 1), which share 25–70% sequence homology. This family of proteins is mitogenic and morphogenic for a variety of cell types (27). The first two members of the family to be purified and characterized were acidic FGF

(FGF-1) and basic FGF (28–31). The core structure of the FGF family is represented by the 18 kDa FGF-2 polypeptide. This structure contains the regions of the molecule that are required for binding to cell surface receptors (32). The N-terminal extensions found on FGF-2 and -3 result from alternative initiation at CUG codons located upstream from the AUG initiation codon (33–35). The nuclear localization sequence contained in the N-terminal extension supersedes the signal peptide in FGF-3, since the higher molecular mass form is targeted to the nucleus, whereas the nonextended form is secreted (33). It is likely that the nuclear localization sequence is dominant over other targeting mechanisms for FGF-2, since the higher molecular mass forms are targeted to the nucleus, whereas the 18 kDa form is preferentially exported to the cell surface (36). Alternative splicing also increases the functional diversity of the FGF family. To date, splice variants have been described for 10 of the 23 FGFs (37–43). In many cases, splicing results in differential subcellular localization of the growth factor, suggesting an additional regulatory mechanism for FGF action.

Although several members of the FGF family are secreted by the traditional endoplasmic reticulum-Golgi pathway, an interesting feature of FGF-1, -2, -9, -11 through -14, -16, and -20 is the lack of a hydrophobic signal sequence in the N-terminus to target the growth factor to the secretory pathway. Despite the lack of a signal peptide, FGF-1, -2, -9, -16, and -20 are known to be secreted (44–48). Secretion of FGF-2 into the extracellular compartment occurs via a nontraditional pathway distinct from the endoplasmic reticulum-Golgi complex (44,49). FGFs can also be released from cells due to lethal or sublethal injury (50,51). Secretion of the growth factor may not be necessary for all of its biological activity, since FGF-2 can act in an intracrine manner by binding to intracellular forms of the receptor (52,53).

3.2. Expression of FGF-2

FGF-2 mRNA is present in astrocytes and several subpopulations of neurons (54,55). FGF-2 protein is also present in astrocytes and neurons *in vivo*, although the mRNA does not always colocalize with protein expression (56). A comprehensive review of the expression of FGF-2 in the rat brain has been published (56). Astrocytes in culture have also been shown to express FGF-2 mRNA and protein (57,58).

Local expression of FGF-2 mRNA and protein are increased following traumatic brain injury (59,60). Ischemia, neuronal degeneration, stimulant drugs, and excitotoxic injury also result in enhanced expression of FGF-2 (61–64). An increase in FGF-2 mRNA is detected as early as 4 h after injury, and persists for at least 14 d, with a peak at around 7 d post-lesion. Expression of FGF-2 protein follows a similar time course. Although FGF-2 is expressed in neurons, microglia, and macrophages, the majority of FGF-2 mRNA and protein is expressed by astrocytes (60). The peak of FGF-2 expression also coincides with the peak of the astrogliotic response.

Expression of FGF-2 is altered with aging. FGF-2 immunoreactivity decreases with advancing age in several regions of the rat brain (65–67). This decrease is not associated with a reduction in the number of GFAP-positive astrocytes. Despite a decrease in the level of FGF-2 expression in the intact brain, systemic administration of glucocorticoids or β -adrenergic agonists results in an increase in FGF-2 mRNA comparable to that seen in young animals, indicating that induction of FGF-2 can occur in the aged brain (68). However, FGF-2 expression following injury to the aged brain has not been examined.

3.2. FGF Receptors

Three broad classes of receptors for FGFs have been identified. High affinity FGF receptors (FGFR) are cell surface receptor tyrosine kinases that bind FGFs with affinities of 10–500 pM. Low affinity FGF receptors consist of cell surface heparan sulfate proteoglycans (HSPG), which bind FGFs with affinities of 2–10 nM. A cysteine-rich FGF receptor (CFR) has also been identified which does not exhibit significant sequence identity with FGFR, and binds several of the FGFs with high affinity (69). The CFR is localized to the Golgi apparatus and plays a role in the intracellular trafficking of FGFs (70). FGFR represents the primary signal transducing receptor for the FGFs.

To date, four distinct FGFR genes have been identified, sharing 55–72% amino acid sequence identity (71,72). Full-length FGFR consists of an extracellular domain with three immunoglobulin-like domains, a hydrophobic transmembrane domain, and a cytoplasmic domain containing a tyrosine kinase domain split by a short insert. All four FGFRs are expressed in multiple forms resulting from alternative splicing (72,73). Although alternative splicing is observed in other growth factor receptor systems, the FGFR system is unique in the vast array of splice variants identified. All four of the FGFRs are known to be activated by FGF-2. Interestingly, the FGFs are not the only ligands capable of binding to the FGFRs. A yeast expression screening method has been used to identify two additional ligands which bind to and activate FGFR1 (74). These ligands are distantly related to the epidermal growth factor and angiogenin families, and show no homology to the FGFs. In addition to soluble factors, cell adhesion molecules (CAM) may bind to and activate FGFR. FGFR1 contains a CAM-homology domain immediately downstream of the acid box (75), and interaction with N-CAM, N-cadherin, and L1 at this site may be responsible for CAM-induced neurite outgrowth (76).

3.3. Expression of FGFR

Early studies suggested that FGFR1 was expressed specifically by neurons, FGFR2 and 3 were expressed by glia, and FGFR4 was not expressed in the CNS (77). However, this localization was based primarily upon the detection of FGFR1 mRNA in gray matter and FGFR2 and 3 mRNA in white matter. More recent studies have demonstrated the *in vivo* expression of FGFR1 mRNA in neurons and astrocytes (56,60), FGFR2 mRNA in neurons, astrocytes, and oligodendrocytes (78,79), FGFR3 mRNA in astrocytes (80), and FGFR4 mRNA in a small population of cholinergic neurons in the medial habenular nucleus (81,82).

Increased astrocyte expression of FGFR1 mRNA has been observed in response to traumatic brain injury (60), and increases in FGFR1 and FGFR2 mRNA expression by astrocytes have been reported in response to transient ischemia (79). Expression of FGFR1 protein in astrocytes is also increased following traumatic injury (Fig. 2). This increase is seen as early as one day following injury and further increases are seen for at least 10 d (83). By 28 d following a traumatic injury, expression of FGFR1 has returned to control levels, except for a sustained upregulation in the glia limitans at the edge of the lesion. The temporal pattern of FGFR1 expression following injury correlates well with GFAP expression, suggesting a potential role for FGFR1 upregulation in astrocyte hypertrophy.

Expression of FGFR1 in the brain, both before and after injury, is regionally heterogeneous. For example, astrocytes in the mid-cortex express lower levels of FGFR1 than

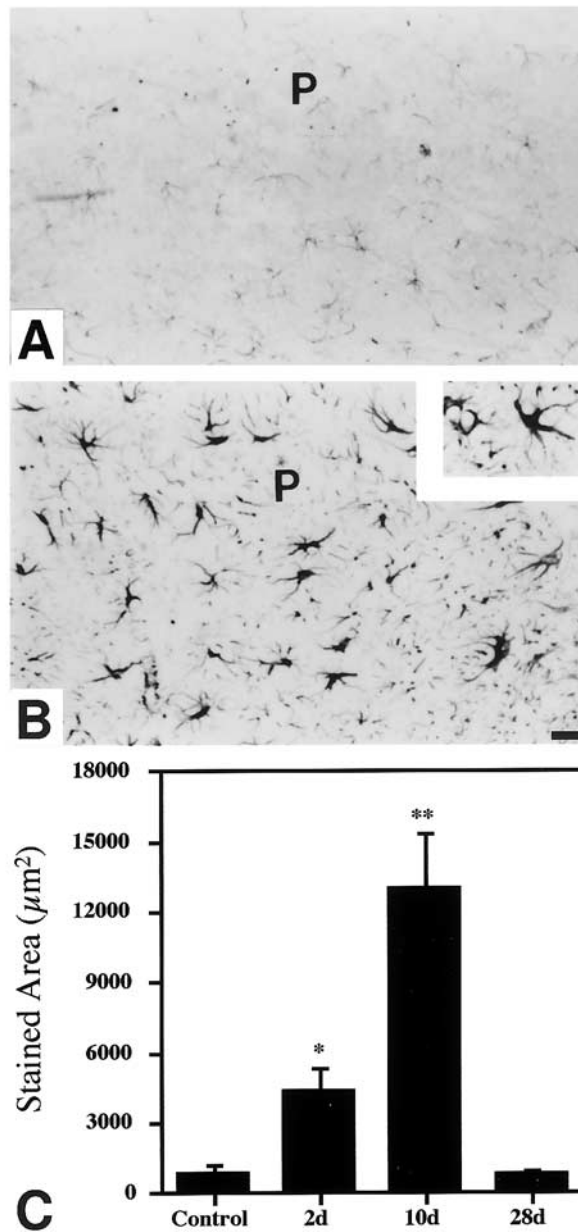


Fig. 2. Hippocampal FGFR1 expression following traumatic brain injury. In nonlesioned animals (A), expression is low. At seven days post-lesion (B), a substantial increase over nonlesioned controls is evident. A high magnification view is shown (inset). Wound edge is to the right of the fields shown; the stratum pyramidale (P) is indicated. Scale bar, 20 μm. Quantitative image analysis (C) of FGFR1 expression after injury demonstrates a 16-fold increase by 10 d post-lesion, with a return to control levels by day 28. (* $p < 0.05$ vs nonlesioned; ** $p < 0.005$ vs non-lesioned). Modified from (83).

those in the hippocampus in the intact brain, but display a substantially greater relative increase following injury (83). This heterogeneity is consistent with differences in astrocyte expression of other proteins (84). These differences may have functional consequences for wound healing, regenerative efforts and the potential for functional recovery. It has been shown that reactive astrocytes can, in certain locations in vivo, permit axonal regrowth following a traumatic brain injury (85).

4. EFFECTS OF FGF-2 ON ASTROCYTE HYPERTROPHY

4.1. *In Vitro* Models

Much of the work on the effects of FGF-2 on astrocytes has been performed using in vitro models. Treatment of cultured astrocytes with FGF-2 enhances cell proliferation in a dose-dependent manner (86,87). Cultured astrocytes also undergo morphological differentiation with FGF-2 treatment, changing from a flat, polygonal morphology to a stellate morphology and elaborating long processes (86,88). In addition to mitogenic and morphogenic effects on astrocytes in culture, FGF-2 enhances the expression of several gene products, including immediate early genes (89), glutamine synthetase (87), and extracellular matrix (ECM) molecules (90,91).

4.2. *Intralesion Injection of FGF-2*

In vivo studies on the effects of FGF-2 on astrocytes have focused primarily on the modification of the astrocyte response to injury. Several studies have demonstrated that FGF-2 enhances trauma-induced astrogliosis (20,92,93). The effects of FGF-2 on astrogliosis primarily represent an increase in astrocyte hypertrophy, as opposed to proliferation. Increased numbers of GFAP-positive cells following traumatic injury are sometimes attributed to astrocyte proliferation. However, studies using ³H-thymidine or bromodeoxyuridine uptake to directly assess proliferation have shown that cell division represents only a minor component of astrogliosis (18,19), and that the increase in astrocyte cell number represents increased detection of astrocytes previously expressing GFAP at subdetectable levels. Direct injection of FGF-2 into a brain lesion increases astrocyte hypertrophy in a dose-dependent manner (20). This increase is detectable as early as two days following injection. Astrocytes in the vicinity of the injection demonstrate enhanced cell size and ramification of processes compared to diluent-injected controls.

In addition to effects on astrocyte hypertrophy, intralesion injection of FGF-2 has effects on other glial cells at the site of injury (Fig. 3). Microglial cells proliferate extensively in response to injection of FGF-2 (20). Although microglia express all four FGFRs (94,95), it is possible that this proliferation represents an indirect response to FGF-2. Pure cultures of microglia do not show a substantial proliferative response to FGF-2 (96), but do proliferate extensively in response to granulocyte-macrophage colony stimulating factor (GM-CSF; 97,98). GM-CSF is produced by astrocytes (99), and is released in response to injury (100), and this may be further enhanced by FGF-2 injection.

4.3. *Co-administration of Heparan Sulfate*

Heparan sulfate (HS) represents the binding component of the low-affinity receptors for FGFs. HSPG is expressed in the ECM of the cortex and hippocampus, and in the

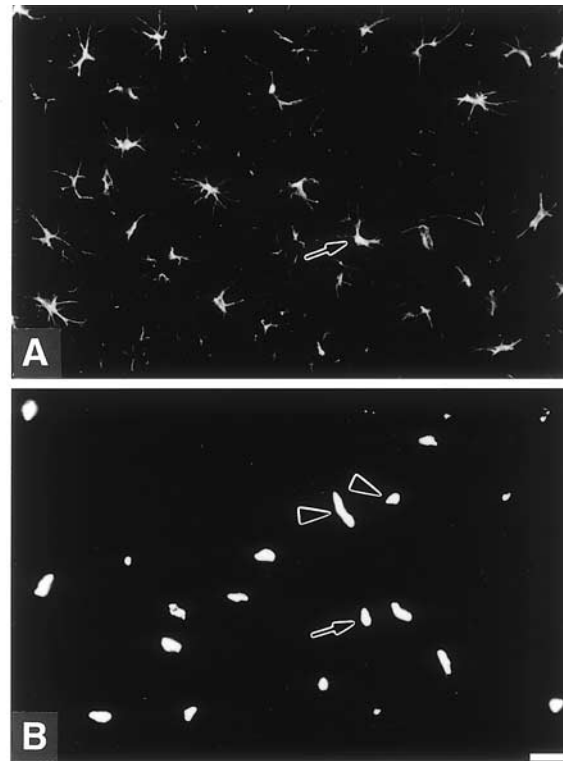


Fig. 3. Immunofluorescent double-labeling for GFAP and bromodeoxyuridine (BrdU) in the cortex of animals injected with FGF-2. GFAP immunoreactivity (**A**) is present in the cytoplasm and processes of astrocytes. BrdU staining (**B**) of nuclei of dividing cells in the same field. Arrows indicate astrocyte processes (**A**) and nucleus (**B**). Nuclei of other dividing cells, primarily of macrophage-microglial phenotype, are also indicated (arrowheads, **B**). Scale bar, 30 μm . From (106).

basal laminae of capillaries throughout the brain (101,102). Although there has been debate in the literature about an absolute requirement for HS participation in the high-affinity binding of FGF to FGFR, HS does seem to facilitate this interaction, perhaps by inducing a conformational change in FGF, or by promoting dimerization of FGF leading to dimerization of FGFR (32,103). In addition to participation in FGF binding to FGFR, HS may serve as a sink for FGF, sequestering free growth factor. Intracerebrally injected FGF-2 exhibits only limited diffusion into the brain parenchyma, as determined by studies using iodinated FGF-2 (104). The growth factor remains bound to HSPG adjacent to blood vessels, and is available only to cells in the immediate vicinity of the injection site. In the injured brain, these low-affinity binding sites are likely to prevent lateral diffusion of FGF released from injured cells or secreted by intact cells.

Several studies have demonstrated that coinjection of HS with FGF-2 into a lesion enhances the glial response (93,105,106). The combination of FGF-2 and HS results in greater astrocyte hypertrophy in the vicinity of the lesion compared to FGF-2 alone. Another important effect of coadministration of free HS is an increase in the radius of growth factor diffusion. In vitro studies have demonstrated that heparin, and to a lesser

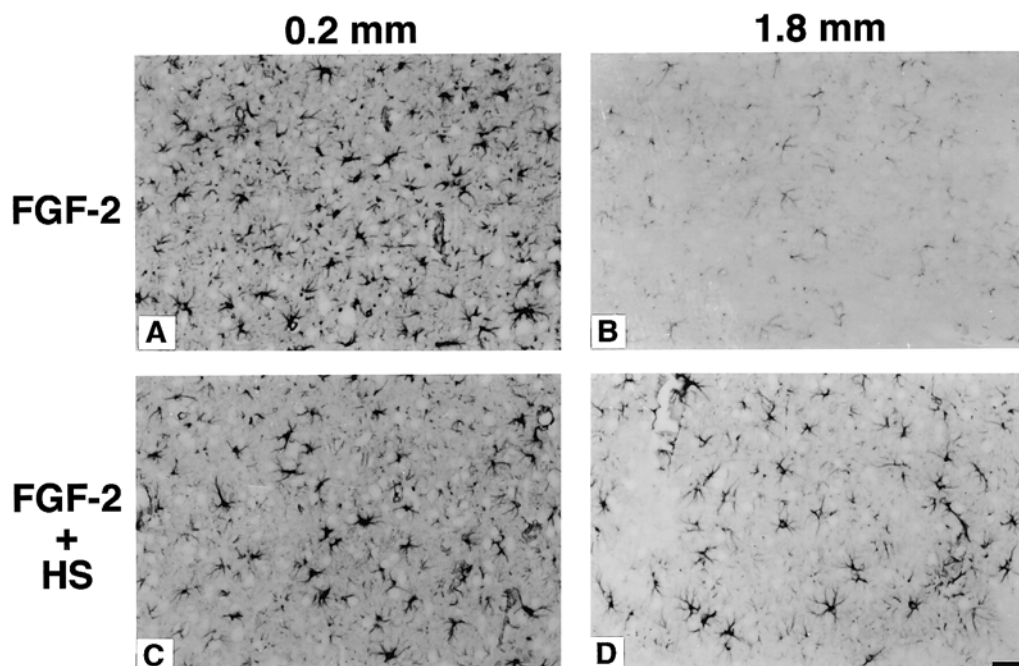


Fig. 4. Immunohistochemical staining for GFAP in following intralésion injection of FGF-2 (A, B) or FGF-2 + HS (C, D). Subfields are centered 0.2 mm (A, C) or 1.8 mm (B, D) from the lesion. Astrocyte hypertrophy is evident at a substantially greater distance from the lesion with coadministration of HS. Scale bar, 30 μ m. Modified from (106).

extent HS, stimulate morphological changes in a monolayer of endothelial cells at a 10-fold greater radius than FGF-2 alone (107). When FGF-2 is injected into a cortical lesion, astrocyte hypertrophy is significantly enhanced within 0.8 mm of the injection (Fig. 4). The combination of FGF-2 and HS produces an increase in astrocyte hypertrophy to a distance of at least 2.8 mm from the injection site (106). This effect may result from HS binding to the low affinity receptor binding sites on FGF-2, allowing the complex to bypass low affinity receptors in the injured tissue, as suggested by Schlessinger et al. (103).

5. EFFECTS OF FGF-2 AND TGF- β 1 ON ASTROCYTE GFAP EXPRESSION

5.1. Transforming Growth Factor- β

In addition to FGF-2, other growth factors and cytokines play a role in the regulation of astrogliosis, including transforming growth factor- β (TGF- β). To date, three TGF- β isoforms (TGF- β 1, - β 2, and - β 3) have been reported in mammals (108). TGF- β 1 is now known to be representative of a large family of growth factors, whose members share 25–90% sequence identity. This family comprises over 40 members, including the inhibins and activins, the bone morphogenetic proteins, decapentaplegic and other *Drosophila* proteins, Müllerian inhibiting substance, the growth-differentiation factors, and glial cell-line derived neurotrophic factor. Members of the TGF- β superfamily exert two major types of effects on most cells: they inhibit proliferation and they strongly activate the production of ECM proteins (109).

In vivo treatment of astrocytes with TGF- β 1 results in increased transcription of GFAP as early as 4 h following growth factor administration (110). Injection of TGF- β 1 into a cortical lesion enhances astrocyte production of laminin and fibronectin and increases glial scarring, whereas neutralizing antibodies to TGF- β 1 reduce ECM production following injury (111). Overexpression of TGF- β 1 in a transgenic mouse model results in increased astrocyte expression of GFAP, laminin, and fibronectin (112). These data suggest that TGF- β 1 is a key regulator of the astrocyte cytoskeleton and the production of ECM components.

5.2. Regulation of GFAP Expression and Process Outgrowth

The effects of FGF-2 and TGF- β 1 on astrocytes could be direct, or secondary to effects on other cells at the injury site. In vitro studies have helped to clarify this issue, and have revealed substantial differences in the response to short- vs long-term growth factor treatment. Pure astrocyte cultures treated for up to 24 h with TGF- β 1 exhibit a rapid and dose-dependent increase in the expression of GFAP mRNA, and this increase is translated into a change in the steady-state level of GFAP protein (113). The increase is detectable within 3 h of treatment onset, and maintained for at least 24 h (Fig. 5). An increase in the initiation of transcription is at least partially responsible for the increase in GFAP mRNA (110), but an alteration in mRNA stability may also be involved. Treatment of astrocytes with FGF-2 results in a rapid and dose-dependent decrease in GFAP expression over a time course similar to that observed with TGF- β 1 (Fig. 5). Treatment with a combination of FGF-2 and TGF- β 1 also results in decreased GFAP expression, suggesting that FGF-2 can inhibit the TGF- β 1-mediated increase in GFAP synthesis.

Although short-term TGF- β 1 treatment results in increased expression of GFAP mRNA and protein, the growth factor has only minor effects on astrocyte morphology (113,114). Conversely, FGF-2 treatment decreases expression of GFAP up to 24 h following administration, yet produces a dramatic morphological change to a process-bearing phenotype (86,88,113). These data demonstrate that astrocyte GFAP expression is uncoupled from process formation. It is likely that the initial events of process formation represent an actin-dependent process (115).

When applied for longer time periods, FGF-2 and TGF- β 1 have effects on GFAP expression very different from those in the short term. Treatment of cultured astrocytes with FGF-2 for 72 h results in a significant increase in the expression of GFAP, while TGF- β 1 treatment has no effect on GFAP expression (Fig. 5C). These effects highlight the complexity of GFAP regulation; following 1 day of in vitro treatment, FGF-2 inhibits a TGF- β 1-mediated increase in astrocyte GFAP expression, while after 3 days of treatment, TGF- β 1 potentiates an FGF-2-induced increase. The data also suggest that the early increase in GFAP expression caused by TGF- β 1 is not sufficient to produce a long-term change in GFAP protein levels or astrocyte morphology. FGF-2 treatment, which induces a rapid morphological response in the absence of increased GFAP expression, also results in a delayed increase in GFAP expression. This suggests that the increase in GFAP expression seen at later time points is necessary to stabilize astrocyte processes elaborated due to FGF-2-induced rearrangements of the actin cytoskeletal network.

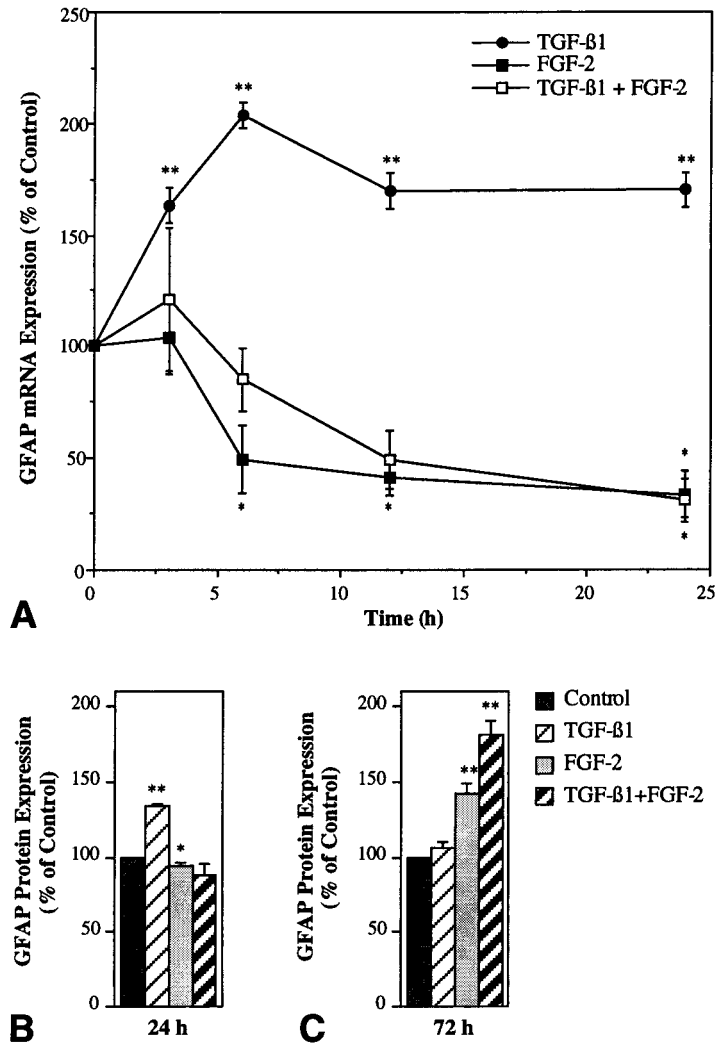


Fig. 5. Effects of TGF-β1 and FGF-2 on GFAP expression. GFAP mRNA expression (**A**) following 3, 6, 12, or 24 h treatment with TGF-β1 (2 ng/mL), FGF-2 (20 ng/mL), or a combination of the two is increased with TGF-β1 treatment and decreased with FGF-2 or the combination of the two growth factors. Changes in GFAP protein expression with 24 h treatment (**B**) follow a similar pattern. After treatment for 72 h (**C**), FGF-2 and TGF-β1 + FGF-2-treated cultures show increases in the expression of GFAP protein, while GFAP protein expression in TGF-β1-treated cultures has returned to control levels. All values are expressed as the percent of control, and represent the mean ± standard error of three independent cultures. * $p < 0.05$ vs control, ** $p < 0.005$ vs control. **A** and **B** modified from (113).

6. ASTROCYTE-DERIVED FGF-2

As discussed in **Subheading 5.2.**, astrocytes express FGF-2, and this expression is upregulated following injury. There is also evidence that FGF-2 induces its own synthesis (116). In addition to effects on astrocytes, FGF-2 is available to act on other cells in the injured area, such as microglia and macrophages. FGF-2 is also a survival factor for

neurons (117–121). Furthermore, FGF-2 has been used successfully in vivo as a neuroprotective agent following traumatic, ischemic, neurotoxic, and excitotoxic injuries (122–124), and has been shown to promote functional recovery in the injured spinal cord (125). Taken together, these data indicate that the role of FGF-2 in astrogliosis extends beyond effects on astrocytes themselves. The upregulation of FGF-2 expression in reactive astrocytes at the site of injury is likely to be part of a generalized trophic response for support of injured neurons. In addition to FGF-2, astrocytes in vivo produce a variety of other growth factors following injury, including nerve growth factor (126), insulin-like growth factor I (127), TGF- β 1 (128), and ciliary neurotrophic factor (129). These growth factors, like FGF-2, have neuroprotective effects (130–132), and their upregulation represents a beneficial aspect of astrogliosis.

7. CONCLUSIONS

Astrogliosis represents a coordinated response to a complex set of stimuli. Injury to the CNS results in changes in the microenvironment including ionic and pH perturbations, breakdown of the blood-brain-barrier, entry of nonneural cells, and the release of a variety of cytokines, growth factors, and other local chemical mediators. The interplay of these factors is a dynamic process, having a variety of effects on all cells in the injured area. FGF-2 induces astrocyte hypertrophy in vivo, and modifies the biochemical responses of astrocytes to injury. In the aged brain, astrocyte responses to injury are altered. It is likely that these alterations result from age-related changes in the expression of growth factors, including FGF-2. Taken together, these data support a significant role for FGF-2 in the regulation of astrogliosis.

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Trophins as Mediators of Astrocyte Effects in the Aging and Regenerating Brain

Judith Lackland and Cheryl F. Dreyfus

1. INTRODUCTION

In their role as cells that provide multiple forms of support to the central nervous system (CNS), astrocytes are beginning to be appreciated as suppliers of critical survival and differentiation factors to neurons and other glia. Current work suggests that astrocytes may express these trophic factors throughout life, not only in the developing fetus, but also in the adult and aged individual. Moreover, studies on brain injury suggest that expression of astrocyte-derived factors is plastic and may be regulated by molecules elicited by injury, such as cytokines, hormones, and growth factors, as well as neurotransmitters. The hope is that as a result of these studies these regulatory molecules may be utilized to enhance astrocyte trophin function and thereby support the aging and injured brain. To examine these contentions, this chapter

1. Defines expression of growth factors by adult astrocytes,
2. Considers the regulation of this expression by injury,
3. Details effects of injury-induced molecules on growth factor expression and
4. Discusses the possible implications of alterations in astrocyte-derived trophic support on learning, memory, and the vulnerability of the CNS to aging and injury.

We focus in this review on astrocyte expression of trophic factors, bearing in mind that astrocytes express a host of other molecules that enhance or diminish neuron function (1,2). In addition, we have limited this review to astrocytes, but it should be appreciated that other glial subtypes, i.e., oligodendrocytes (3) and microglia (4) also express survival factors and they too undoubtedly contribute to the support of neurons, as well as astrocytes, in CNS function.

2. EXPRESSION OF GROWTH FACTORS BY ASTROCYTES IN THE ADULT BRAIN

2.1. *The NGF Gene Family*

That trophic factors are expressed by astrocytes was first suggested 20 yr ago by identification of nerve growth factor (NGF) in astrocytes in culture. In these studies, conditioned medium (CM), derived from lesioned adult rat brain astrocytes, was found

to enhance survival of NGF-responsive, as well as NGF-insensitive, peripheral neurons (5). Effects of CM on NGF-dependent neurons were blocked by neutralizing antibodies to NGF, supporting the claim that at least some of these effects could be attributed to NGF. These observations were confirmed by others who found NGF protein and mRNA were expressed by cultured neonatal mouse and rat brain astrocytes and that NGF was in CM derived from these cells (6–13). Not only was NGF present in these astrocytes, the expression appeared to be regulated by growth status; proliferating cells expressed more NGF than quiescent ones (7).

These findings prompted other studies that investigated glial NGF mRNA expression *in vivo* and evaluated how expression changes as the brain ages. The optic nerve provided a glial population for such *in vivo* examination. Optic nerves from neonatal and adult rats were monitored for NGF mRNA using nuclease protection assays. NGF mRNA was found to be decreased in the optic nerve from adult as compared to neonatal animals, suggesting that, as in culture, NGF mRNA *in vivo* may be highly expressed in young animals in which glial cells are known to be proliferating. When glia become quiescent, such as in older animals, NGF mRNA decreases (14).

However, these studies of optic glia, although exciting, were limited. The optic nerve is composed of multiple glial subtypes, including oligodendrocytes as well as astrocytes. Study of this model did not address the question of NGF expression specifically in astrocytes. To do so, it was necessary to evaluate relative levels of NGF mRNA or protein in glial fibrillary acidic protein-positive (GFAP+) astrocyte cells. Therefore, *in situ* hybridization studies were performed that examined coexpression of NGF mRNA with GFAP. The findings indicated that NGF mRNA was present at low levels in adult astrocytes of the basal forebrain, neocortex, amygdala, cerebellar cortex, and hypothalamus *in vivo* (15).

Subsequent work examined how such expression of NGF may be regulated during maturation. In particular, cultures were utilized in which cortical astrocytes derived from fetal, neonatal or adult animals were isolated and examined for NGF mRNA. The astrocytes were found to express progressively lower levels of NGF mRNA and protein as the age of the animal from which the astrocytes were derived increased (11). As the brain ages, then, NGF in astrocytes appears to decline.

NGF is a member of a gene family, known as the neurotrophins that includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). Although little is known about NT-4 in astrocytes, BDNF and NT-3 have been found to be expressed by astrocytes. For example, BDNF and NT3 mRNAs were found to be expressed in astrocytes in culture (10,13,16–18) and BDNF, as well as NT3 proteins were localized to astrocytes and astrocyte-like cells *in vivo* (19–21). Moreover, neurotrophin expression was found to be decreased during brain maturation. When astrocytes from the cortex were dissociated from brains of animals of increasing ages and grown in culture, BDNF mRNA expression decreased as animals matured from fetus to adult (personal observation). As a group, then, NGF, BDNF, and NT-3 are expressed by astrocytes and are downregulated during brain maturation.

2.2. Other Growth Factors

Although the neurotrophins have been examined most extensively, other trophic factors have now been found to be produced by astrocytes. These include ciliary neu-

rotrophic factor (CNTF) (10,22,23), fibroblast growth factor-2 (FGF-2) (24–26), enkephalin, somatostatin (27), and glial cell line-derived neurotrophic factor (GDNF) (28,29). Interestingly, *in vivo* studies indicated that while expression of some of these growth factors, for example, FGF-2, decreased *in vivo* with maturity (30), expression of others, such as CNTF, increased with age (31). Moreover, culture studies indicated that neuropeptides were also regulated in an age-dependent manner. For example, cerebellar astrocytes exhibited an increase in expression of proenkephalin and a decrease in somatostatin when derived from progressively older animals (32). Astrocytes, then, appear not only to express multiple factors, but also to alter their roles as trophin providers during maturity by exhibiting selective regulation of the different factors. Relative levels of individual neurotrophins, as well as FGF-2, CNTF, somatostatin and proenkephalin change as aging occurs.

3. EFFECTS OF INJURY ON ASTROCYTIC EXPRESSION OF GROWTH FACTORS

A new literature has indicated that the *in vivo* expression of astrocyte-derived molecules, as defined in the CNS above, is plastic and can be regulated by environmental signals. This has been most clearly demonstrated by studies examining the effects of injury on profiles of growth factor expression. For example, in the adult, a lesion of the optic nerve resulted in enhancement of NGF mRNA expression (14), and chronic neurotoxic damage to the basal forebrain and hippocampus (15), or trauma to the cortex (33) led to an increase in NGF mRNA in GFAP+ cells. Ischemia also led to an increase in NGF protein expression in astrocytes in the hippocampus (34). Studies in culture complemented these *in vivo* findings. When compared with astrocytes from unlesioned animals, NGF protein and mRNA were markedly elevated in cultures of striatal astrocytes derived from brain lesioned by treatment with 6-hydroxydopamine (6HD) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). These astrocytes from the lesioned brain morphologically resembled reactive astrocytes *in vivo*. GFAP mRNA and protein were elevated as a result of the lesion along with vimentin and two epitopes that are only expressed *in vivo* on reactive astrocytes (35,36).

Injury could similarly affect other growth factors. For example, FGF-2 mRNA and protein were found to be increased in GFAP+ astrocytes (37–39) or in astrocyte-like cells (40,41) following damage. Increases in CNTF mRNA, in concert with increases in GFAP+ astrocytes (22,42), as well as increases in IGF-1 mRNA in GFAP+ astrocytes (43) have also been reported.

Importantly, not all astrocyte-derived factors are elevated by injury. For example, *in vivo* studies that indicated that CNTF was increased, demonstrated that BDNF and NT-3 were slightly decreased after injury (22) and culture studies that utilized lesioned astrocytes, showed that NGF mRNA was elevated although proenkephalin was decreased (35). Thus, trophin production in astrocytes is plastic and can be altered by lesions in a selective manner.

The studies of injury have prompted analyses of the molecules that are regulated by injury. These experiments have suggested signals that may underlie plasticity in the aging brain. These include cytokines, hormones, neuronal growth factors, and possibly neurotransmitters (Fig. 1). New studies have begun to explore the effects of these molecules on astrocytes.

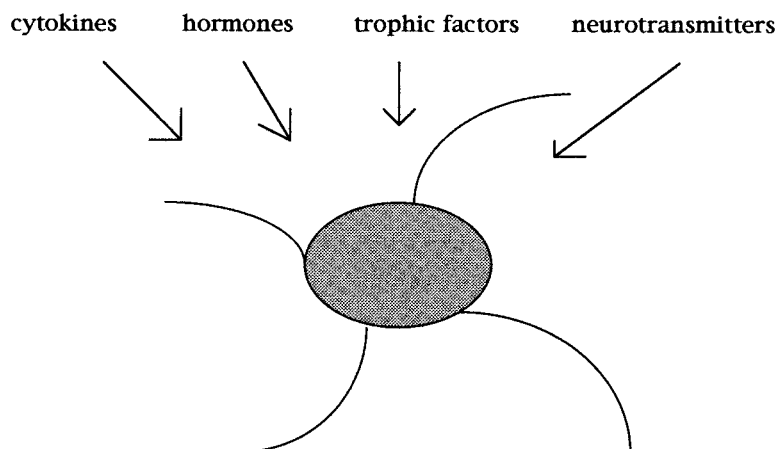


Fig. 1. In the maturing brain astrocytes express receptors for, and respond to, cytokines, hormones, trophic factors and neurotransmitters.

4. REGULATION OF ASTROCYTE GROWTH FACTORS BY MOLECULES ELEVATED FOLLOWING LESIONS

4.1. Cytokines

To begin to explore injury-induced molecules, cytokines, such as interferon-gamma (IFN- γ), interleukin 1 β (IL-1 β), and transforming growth factor- β 1 (TGF- β 1) are being investigated (44–46). These molecules originate in the brain from microglia and astrocytes, or they enter the brain through a disrupted blood-brain-barrier. TGF- β 1, for example, is abundant in platelets that enter the brain after specific lesions (47).

What are the effects of these cytokines on growth factor expression in maturing astrocytes? To address this issue a growing literature has emerged primarily based on studies not of the aging or adult brain, but rather of cultures of early postnatal astrocytes. It has been relatively easy to look at direct effects of cytokines on expression of specific trophins in such neonatal models. For example, neonatal astrocytes have been shown to exhibit increased levels of NGF mRNA in response to bacterial lipopolysaccharide, a potent activator of the inflammatory response (48,49) and NGF mRNA was also found to be elevated in response to multiple cytokines, including IFN- γ (11), TGF- β 1 (13,50–53) and IL-1 β (11,13,52,53). The mechanisms underlying these responses are being elucidated (54,55).

By extension of the work on early postnatal cells, new studies also have begun to evaluate adult astrocytes. Notably, adult cells in culture have been found to respond differently to cytokines than do neonatal cells. For example, adult astrocytes responded only minimally to IL-1 β and IFN γ , whereas neonatal cells exhibited dramatic increases in NGF mRNA expression (36). Lesions, however, modulated responsivity in the adult cells. Cultured adult astrocytes, derived from 6-HD lesioned brains, exhibited a marked elevation of NGF mRNA in response to cytokines (36). These observations suggest that injury not only elicits an increase in molecules that regulate neurotrophin expression, but also sensitizes the cells to cytokines, thus augmenting the response.

As is the case with injury in general, all astrocyte-derived growth factors are not similarly regulated by cytokines. For example, although NGF, as well as FGF-2 expression was elevated by inflammatory molecules (25), BDNF, NT-3, NT-4, GDNF, and CNTF expression was not (13,25). Thus, in response to a lesion, cytokines elicit elevation of a precise combination of growth factors that may be released into the microenvironment to influence nearby neurons and other glial cells. These cytokines are prime candidates for further examination of molecules that regulate astrocytes in the aging brain.

4.2. Glucocorticoids

In addition to altering cytokine levels, brain damage elicits marked increases in glucocorticoid hormones (56,57). Interestingly, these increases also occur during aging (58). The elevated glucocorticoids may have direct effects on astrocytes. Glucocorticoid receptors are expressed in these cells (59,60). Studies of postnatal hippocampal astrocytes revealed that dexamethasone regulated NGF mRNA expression (51,61). Moreover, influences of glucocorticoids on individual growth factors have been found to differ; for example glucocorticoids increase FGF-2 mRNA in the same region where NGF mRNA is decreased (61).

Elevation of multiple factors as a result of injury leads to interaction among these factors that influence astrocyte function. Thus, glucocorticoids have been shown to interact with cytokines, leading to a greater susceptibility to stress. For example, corticosterone inhibited the stimulatory effects of TGF- β 1 and IL-1 β on NGF expression in an astrocyte cell line (62). Interestingly, another steroid, 1, 25-dihydroxyvitamin D3 (calcitriol), potentially produced by microglia (63), stimulated NGF mRNA expression in an astrocyte cell line (62) and in cultured astrocytes (64). Moreover, it augmented the effects of IL-1 β and TGF- β 1 (62). Thus, immune responses to injury are complex and involve interactions of disparate molecules that have the potential to elevate as well as reduce trophin expression. Knowledge of these interactions is important in identifying signals that regulate aging astrocytes, even in the absence of injury.

4.3. Gonadal Steroids

Recent observations have predicted that another class of steroids, the estrogens, also affect growth factor expression in astrocytes. Estrogen response elements have been reported to be present in NGF and BDNF genes (65,66) and these genes have been shown to be regulated by estrogen in neurons *in vivo* (65–68). Moreover, estrogen receptors have been identified in astrocytes and estrogen effects on morphology of postnatal cells in culture and adult cells *in vivo* have been described (69–71). Current work, therefore, has examined effects of estrogen on astrocytes derived from postnatal and adult animals. When estradiol was added to enriched cortical astrocyte cultures, it elevated expression of BDNF mRNA in both adult and neonatal cells. In contrast, NT-3 was elevated in adult, but not neonatal cells (72). Molecules other than neurotrophins, moreover, may be regulated by estrogens. For example, IGF-I in astrocytes appeared to be affected by estradiol *in vivo* (73).

It remains to be determined how responsivity to estrogen may be affected by injury and how estrogen may interact with glucocorticoids or cytokines to influence astrocyte function. However, it is interesting to note that in astrocytes the estrogen synthetic

enzyme, aromatase, and aromatase activity were upregulated following a lesion (74). These cells normally produce little or none of this protein (75). Therefore, CNS injury may result in an increase in the local astrocyte concentration of estrogen in the damaged region.

4.4. Effects of Astrocyte-Derived Growth Factors

As noted in **Subheading 4.3.**, expression of astrocyte growth factors (neurotrophins, FGF-2, and CNTF) decreased or increased as the brain aged or following a lesion. This, potentially resulted in a change in the trophic factor milieu. Effects of such factors on neurons have been examined in some detail. In contrast, the effects on astrocytes have been relatively unknown, but are beginning to be explored.

4.4.1. The Role of Neurotrophins on Astrocytes

In the case of the neurotrophins, data supporting a role for these factors on astrocytes is sparse. This is due in part to the lack of evidence that functional neurotrophin receptors are expressed on astrocytes and the expectation, therefore, that neurotrophins would play no role. Recent studies, however, have begun to force a reexamination of this issue.

Neurotrophin receptors consist of two transmembrane glycoproteins, the p75 neurotrophin receptor and the trk receptor tyrosine kinases (76,77). The p75 receptor has been known as the common neurotrophin receptor because it binds all neurotrophins with equal affinity (78). Astrocytes express the p75 receptor in culture (79–81) and in the adult brain in vivo (81,82) and this expression has been reported to be elevated following a lesion (83). However, the role of the p75 receptor on astrocytes is unclear, particularly in response to damage or aging.

Trk receptors, in contrast to p75, are relatively specific in action. NGF binds most specifically to trkA, BDNF and NT-4 to trkB and NT3 to trkC. These receptors have been reported to be expressed on astrocytes in both full-length and truncated form (79–81). The predominant form, however, is the truncated one, considered to be unable to transduce a neurotrophin signal as measured by immediate early gene induction or by protein tyrosine phosphorylation (81). Recent studies, however, have suggested that these receptors are functional. Truncated trkB receptors were found to bind and endocytose BDNF which could be released as a bioactive molecule to influence neuron survival (84). Moreover, truncated trkB, transfected into a specific cell line, mediated effects of BDNF on the rate of acidic metabolite release (85). These findings have been complemented by observations that the truncated receptor was elevated in response to IFN- γ (84), a molecule upregulated by injury, suggesting that the effects of neurotrophins on astrocytes may be enhanced following injury.

Supplementing the observation that astrocytes express neurotrophin receptors, are the demonstrations that neurotrophins affect astrocytes. For example, it had been shown that NGF regulated levels of p75 (79,80,86) and trkB mRNA (80) in cultured cells. NGF also elicited dramatic increases in astrocyte cell numbers in embryonic septum (Fig. 2) and substantia nigra, as well as alterations in astrocyte morphology (Fig. 3) (87) *see also* (86). The findings that both receptors and ligands are present in the same cells in the adult brain suggests that astrocytes may, through neurotrophins, signal to themselves or neighboring cells to enhance function, not only during development, but also in maturity.

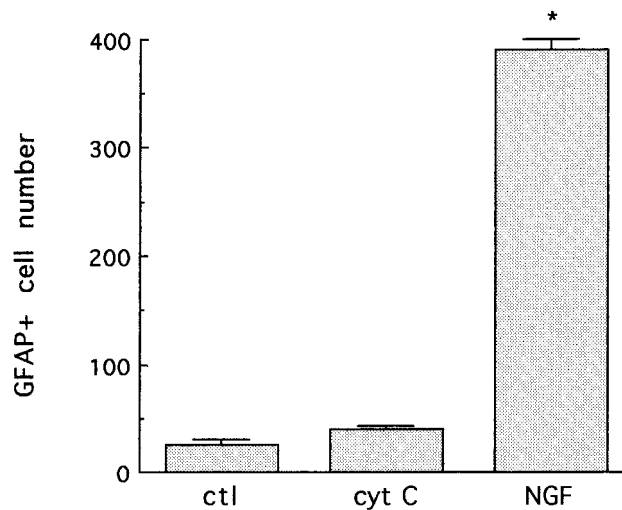


Fig. 2. Effect of NGF on GFAP-positive cell numbers in embryonic septal culture. Cultures were grown for 7 days in the presence (NGF; 1-2ug/ml) or absence (ctl) of NGF or cytochrome C (cyt C; 1-2ug/ml), a compound similar in structure and physicochemical properties to NGF. NGF was prepared from adult mouse salivary glands. NGF from a different preparation (Boehringer Mannheim) also increased astrocytic cell number, indicating that the effect was not due to a potential contaminant in any single preparation. * $p < 0.0001$, ctl vs NGF and cyt C vs NGF. Reprinted with permission (87).

4.4.2. The Role of Other Trophic Factors on Astrocytes

An emerging literature suggests that other trophic factors expressed by astrocytes also affect astrocytes. FGFs, for example, have been reported to elevate expression of NGF in astrocytes (46,47). Moreover, lesions may enhance this FGF action. For example, expression of the FGF receptor *flg*, present on adult astrocytes, was found to be upregulated following injury (35,83). In addition, cytokines, that are increased by a lesion, enhanced actions of FGF-2 (47), supporting the concept that lesions elicit enhanced responsiveness. CNTF has also been reported to affect astrocyte function. For example, it increases GFAP and induces hypertrophy (84). Here, too, injury appears to enhance responsiveness. For example, receptors for CNTF were upregulated on astrocytes by damage, in some cases from almost undetectable levels (25,81). This suggests that following injury astrocytes became further sensitized to the very molecules they themselves express.

4.5. Other Factors that Influence Astrocyte Function: The Role of Neuronal Signals

Increasing reports have indicated that astrocytes express a variety of K^+ channels (90). Moreover, they exhibit α - (91) and β - (92) noradrenergic receptors, dopaminergic receptors (93), muscarinic (94,95), and nicotinic (96) cholinergic receptors, non-NMDA ionotropic and metabotropic glutamatergic receptors (97–100), and peptidergic receptors (93,101). The findings that many of these are present on astrocytes throughout life (95,102–104) and that they are, in some cases, upregulated following injury

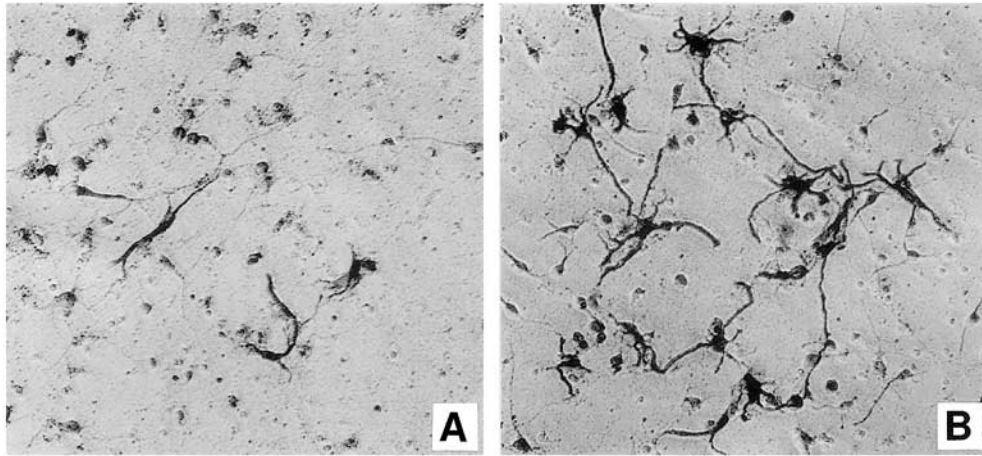


Fig. 3. Astrocytes visualized in dissociated septal cultures using GFAP immunocytochemical procedures. Astrocytes were detected in cultures grown in the absence (A) and presence (B) of NGF (1 $\mu\text{g}/\text{mL}$). Reprinted with permission (87).

(105), indicate that neuronal signals may regulate astrocytes into adulthood and old age. Moreover, astrocytes have been shown to express specific receptors in close apposition to appropriate axons in vivo (92), and K^+ is released from axons at locations in close proximity to astrocytes (106,107), suggesting that astrocytes are in position to respond to neuronal signals. These observations have been complemented by the finding that treatment of astrocytes with appropriate ligands (108–112), or with K^+ (18,113) elicited increases in $[\text{Ca}^{2+}]_i$ and in some cases altered astrocyte morphology.

To begin to determine whether astrocyte response to neuronal signals may extend to effects on trophic expression, effects of norepinephrine and other β -adrenergic agonists on NGF have been evaluated. Norepinephrine and other β -adrenergic agonists found to alter astrocyte expression of NGF (114–116) and BDNF (13), and increase NGF secretion in whole brain astrocytes (114). Dopamine and epinephrine also were to increase NGF and BDNF secretion (8). Moreover, effects of β -adrenergic agonists on astrocytes were not limited to those on neurotrophins. Expression of proenkephalin, somatostatin (11,115) and FGF-2 (117) mRNAs and proteins was enhanced by these ligands. It should be noted, moreover, that all catecholamine effects were not stimulatory. CNTF mRNA and protein as well as FGF-1 mRNA were downregulated in astrocytes that exhibited enhanced expression of NGF or FGF-2 mRNAs and proteins (116–118).

Glutamate also was found to influence growth factor expression. For example, glutamate enhanced expression of BDNF and NGF in the basal forebrain and cortex (17,119), as well as FGF-2 in cortical astrocytes (17), and GDNF in striatal astrocytes (120).

What is the meaning of these responses to neuronal signals for the aging and lesioned brain? To the best of our knowledge, little is known of how neurotransmitter actions on astrocytes may be modified during development and after damage. It is clear that specific neurotransmitter receptors are located on adult astrocytes, suggesting that

astrocytes are sensitive to this type of stimulus throughout life. Furthermore, astrocytes of damaged brain also have been reported to express neurotransmitter receptors (95) that may be upregulated in comparison to unlesioned brain (105). However, undoubtedly the modulation of sensitivity to neuronal signals with aging and in response to lesion again depends on the specific stimulus, the brain region being examined and the responding growth factor being assessed. We have, for example, grown astrocytes from postnatal basal forebrain in culture and compared these to astrocytes derived from the adult. Although neonatal astrocytes responded to K⁺ and glutamate by increasing expression of neurotrophins, adult basal forebrain cells did not, suggesting that responsiveness to these signals decreases as aging proceeds (121). However, it should be remembered that astrocytes may modulate responsiveness to one set of stimuli in the presence of another set of stimuli. It remains to be determined whether the adult basal forebrain astrocytes increase responsiveness to neuronal signals in the presence of the other categories of molecule associated with lesions.

5. CONSEQUENCES OF AGING ON LEARNING, MEMORY, AND VULNERABILITY TO LESION: THE POTENTIAL ROLE OF ASTROCYTES

5.1. Effects of Aging on Brain Function and Trophin Expression

As an animal ages, well documented alterations have been reported to occur in the morphology of neurons and astrocytes. For example, a loss of neurons and dendritic processes has been described that was accompanied by astrogliosis, characterized by elevated GFAP staining, astrocyte hypertrophy, and an increase in the fibrous character of astrocytes (122–124). As a result of these changes, it has been suggested that there is an alteration in the neurotransmitter composition of the brain and that this may underlie decreases in cognition (125). It has further been suggested that neuronal and glial alterations are associated with a decrease in trophic factor expression (126–129). However, when examined, these changes in trophin expression have been found to be variable. Such modulation of trophins has been evaluated most extensively in the basal forebrain-hippocampal system where, in some cases neurotrophins (129) and their receptors (128) were reported to be decreased with aging. In contrast, other studies have found no change in expression (126–128), or an increase in expression (129). Interestingly, in spite of the variability in reports of effects of aging on neurotrophin expression, administration of exogenous NGF resulted in an improvement in cognitive function (130–133) and neuronal function, suggesting that some deficit in trophin expression was indeed present in aging that was, at least partly, corrected.

In an analogous situation, deficits in behavior and neuronal function in aged animals were associated with the nigro-striatal system. Levels of GDNF mRNA were not altered in the striatum of the aged animal (134). However, as with the basal forebrain-hippocampus, both behavioral and neuronal deficits were improved by administration of GDNF (135,136).

The deficits associated with aging extend to responsiveness to injury and stress. For example, aged animals exposed to partial hippocampal lesions, exhibited greater decreases in cholinergic neuron size and choline acetyltransferase staining than did young rats (137). This increase in vulnerability may have been due to a deficit in trophin response to the injury. When gelfoam was used to capture trophic activity that

accumulated following lesion of the striatum, trophic activity derived from aged animals exhibited reduced capacity to support neurite outgrowth in culture than did trophic activity accumulated from younger animals (138). In other studies, trophic activity in lesioned aged brains took longer to attain maximal activity when evaluated in culture than did activity in young brain (139).

Stressed rats similarly appear deficient in trophin response. Changes in BDNF expression in response to stress were attenuated in the aged animal. Moreover, these changes were specific and were not noted with respect to NGF or NT-3 (140).

The molecules underlying these alterations in responsiveness have begun to be delineated. In the denervated striatum, for example, interleukin 1 expression has been reported to be markedly reduced in older rats as compared to younger animals (141). The literature reviewed in this chapter gives hints as to what other molecules may be altered as aging progresses.

5.2. Involvement of Astrocytes in Deficits Associated with Aging

Data on the aging brain imply that aging may be accompanied by deficits in trophin expression that lead to increased neuronal vulnerability. What role do astrocytes play in this deficit and can we ultimately make use of these cells to counteract loss?

An emerging literature suggests that astrocytes from the aging brain do indeed exhibit impairments in function. For example, astrocytes grown for prolonged time in culture were reported to be deficient in their ability to respond to oxidative injury (142). In another model of aging, astrocytes passaged for 46–51 passages exhibited diminished ability to respond to microenvironmental signals (143). We (121) and others (36) have found that astrocytes derived from adult brain expressed reduced levels of mRNA encoding trophic molecules and a diminished response to neurotransmitters or cytokines. In vivo, glia of aging animals demonstrated reduced expression of specific trophic molecules (14).

This chapter indicates that astrocytes express multiple trophic factors that may contribute to the milieu of the aging and injured brain. The expression of these trophic molecules, moreover, is plastic, and is regulated by cytokines, hormones, growth factors and neurotransmitters. Following a lesion, and as aging progresses, astrocytes have been widely reported to increase in number and size relative to neurons and they emerge as prime candidates to target in order to reverse deficits in trophin production that may be associated with aging and responsiveness to injury.

Future studies must make use of the current culture models, complemented by in vivo studies, to further determine events modulation astrocyte function in aging and injured animals. The excitement of the field is the hope that with extension of the information currently available, we may be able to manipulate the complex variety of factors that are produced by astrocytes to ameliorate the deficits associated with aging and disease.

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Responses in the Basal Forebrain Cholinergic System to Aging

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1. INTRODUCTION

Documentation of age-related mental deficiency, described as the decline and decay of the human body and regression of mental capacities by 63- and 81-yr-of-age, dates to the description of the life cycle by Pythagoras, a Greek physician of the 7th century B.C. He described the late stage of life where “*the scene of mortal existence closes, after a great length of time, ... The system returns to the imbecility of the first epoch of the infancy.*” The more modern description of senile dementia dates to Alois Alzheimer’s description in 1906 of the first documented case of Alzheimer’s disease (AD) (1). During the last quarter of this century there has been significant progress in the fields of neuropathology, biochemistry, and genetics, as well as some pharmatherapeutic interventions aimed at this dementing disorder. However, there is no definitive characterization of the mechanisms of this disease (2).

Alzheimer’s disease, the most common dementing disorder of late life and a major cause of disability and death in the elderly, is manifested by behavior deficits involving memory and cognition, accompanied by personality changes that increase in frequency with advancing years. Alzheimer’s disease is associated with an excessive loss of synapses and mainly cholinergic neurons in specific brain regions, deficits in neurotransmitter function, and marked extracellular deposition of amyloid deposits that form senile plaques and the prominent appearance of intracellular neurofibrillary tangles [for reviews, see (3,4,5).] Although to a lesser extent, there are similar changes evident in the healthy aged brain (6). Also, neurotransmitter- and/or amyloid precursor protein (APP)-mediated disruptions of signal transduction cascades may exacerbate AD pathology (7).

The appearance of a link between cholinergic dysfunction and AD has focused scientific efforts on developing tools that elucidate the regulatory events controlling cholinergic system function with the goal of developing therapeutic interventions for the disorder. An important step in understanding the mechanisms underlying cholinergic dysfunction has been the development of *in vivo* rodent models that mimic some features of AD. Using such *in vivo* rodent models, several cholinergic enhancement strategies have been tested that have proven to be somewhat effective in alleviating

lesion-induced cognitive deficits (8). These include acetylcholine precursor loading approaches (e.g., lecithin); treatment with postsynaptic agonists, acetylcholinesterase inhibitors (e.g., tacrine and donepezil), neurotrophic factors (e.g., nerve growth factor); and transplantation of cholinergic-enriched fetal grafts.

2. CHOLINERGIC DEFICITS IN THE AGED AND IN ALZHEIMER'S DISEASE

2.1. *The Cholinergic Basal Forebrain*

Cholinergic neurons are those that produce, store, and release acetylcholine (ACh), the first substance recognized to be a neurotransmitter. Choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), are the two rate-limiting enzymes that regulate together with choline uptake, ACh synthesis, and degradation. Thus, measurement of the specific activities of ChAT and AChE are useful markers for cholinergic function. Cholinergic neurons are medium to large sized cells (range, 18–43 μm in maximum soma extent) with long axons that can be identified by histochemical staining with AChE, immunocytochemistry for ChAT, and *in situ* hybridization for ChAT mRNA (9,10,11,12). In the brain, cholinergic neurons are mostly localized within the basal forebrain and to a lesser extent in the brainstem and cerebellum.

The basal forebrain cholinergic system in the adult contains three major subnuclei of projection neurons within the medial septum (MS), the diagonal band of Broca (DB) and the nucleus basalis of Meynert (NBM), along with interneurons of the striatum. Anatomical tracking studies (13) have shown that cholinergic basal forebrain neurons (CBFNs) give rise to a dense network of cholinergic fiber projections. The basal forebrain cholinergic neurons project from:

1. the NBM to the cerebral cortex and amygdala, bundling as the basalo-cortical and basalo-amygdalar afferents;
2. the MS and vertical limb of DB to the hippocampus via the fimbria-fornix forming the septo-hippocampal afferents;
3. the lateral portion of the horizontal limb of DB to the olfactory bulb and associated nuclei (9) (Fig. 1).

There are also noncholinergic neurons intermingled with the CBFNs within or surrounding the basal forebrain, some of which are GABAergic septal neurons that contain parvalbumin (14,15); and substantia innominata neurons that contain calbindin-D_{28K} or NADPH-diaphorase (16). CBFNs with extensive dendrites also intersect with other cholinergic and noncholinergic neurons within the basal forebrain extending into and intertwining with heavily myelinated fibers. In the hippocampus and cortex, pyramidal neurons appear to constitute the primary synaptic target for CBFNs (12).

That there is a relationship between cholinergic function and memory and learning was initially ascertained from observations of aged and demented patients in their responses to various pharmacological manipulations. Application of the muscarinic receptor antagonist, scopolamine, to young normal subjects produces deficits in cognition and memory that are similar to those seen in aged subjects. Additionally, administration of the cholinergic agonist, physostigmine, to aged human subjects results in an improvement in memory function (17,3,18). Neuropsychological, biochemical, and

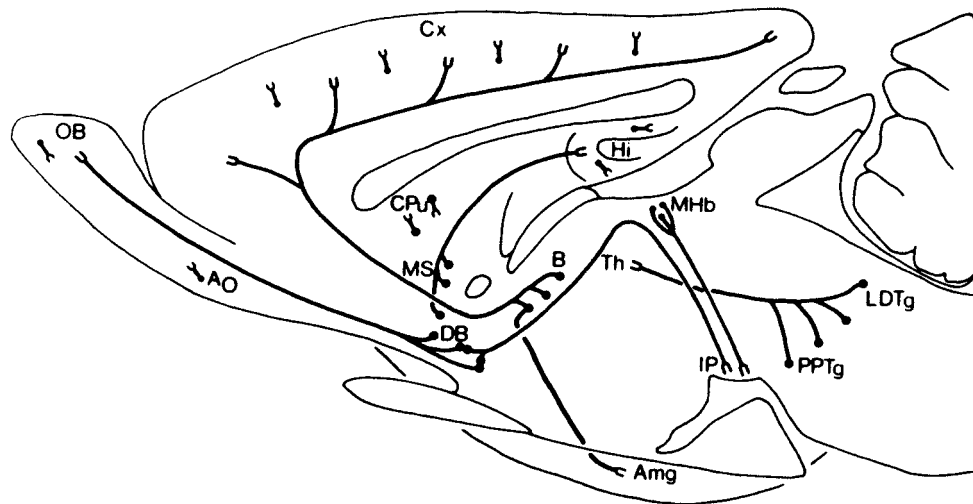


Fig. 1. Schematic illustration of the horizontal plane of the major cholinergic systems in the rat brain. The basal forebrain cholinergic system contains three major subnuclei of projection neurons within the medial septum (MS), the diagonal band of Broca (DB) and the nucleus basalis of Meynert (B), along with interneurons of the striatum. The basal forebrain cholinergic neurons (CBFNs) project from: (1) the nucleus basalis of Meynert to the cerebral cortex (Cx) and amygdala (Amg), bundling as the basalo-cortical and basalo-amygdalar afferents; (2) the MS and vertical limb of DB to the hippocampus (Hi) via the fimbria-fornix forming the septo-hippocampal afferents; and (3) the lateral portion of the horizontal limb of DB to the olfactory bulb (OB) and associated nuclei. Modified from Butcher, et al. (10).

pharmacological evidence further supports the notion of a significant role for cholinergic function in age-related memory disturbance leading to a proposal of a cholinergic hypothesis of dementia in aging and age-related disorders.

2.2. Cholinergic Deficits in Aging and Alzheimer's Disease

The presence of cholinergic deficits and the loss of CBFNs in the aged and AD brains have been well documented (19,20,21). Postmortem studies have shown that there is a profound reduction in cortical presynaptic cholinergic markers in patients with AD and senile dementia of the Alzheimer's type. For example, ChAT activity decreases 60–90% in the cerebral cortices and hippocampi of AD patients. The cholinergic neurons of the NBM, the major source of cortical cholinergic innervation, undergo a profound (greater than 75%) and selective degeneration in these patients and the degree of loss correlates with the severity of the observed cognitive impairments (21,22). In addition, an *in situ* hybridization study (23) has shown decreased ChAT mRNA expression in the NBM in AD patients, suggesting that expression of ChAT mRNA might be downregulated in surviving cholinergic neurons. Reductions are also observed in high-affinity choline uptake (HACU) (24), ACh and AChE levels in cortex (25) and cerebrospinal fluid (CSF) (26). Nerve growth factor (NGF) receptor and ChAT remain colocalized in the NBM in AD patients (27). Apart from the presence of cholinergic dysfunction, there are (28) moderate increases in NGF-like activity

throughout the brain of AD coupled with significant declines in NBM cell numbers compared to aged healthy individuals. Although the mechanisms that lead to the degeneration of cholinergic neurons in the NBM are unknown, it has been speculated that neuronal death may result in part due to a failure of neurotrophic support for the maintenance of oxidant-antioxidant and glutathione peroxidase homeostasis (29,30,31).

There is a substantial decrease in ChAT activity in the striatum, and there are decreases in HACU in the frontal cortex and hippocampus, of 24-mo-old rats when compared to 4-mo-old rats (32). However, Ogawa, et al. (33) found that reduced ChAT activity and muscarinic M1 receptor levels in aged Fisher 344 rat brains did not parallel their mRNA levels, suggesting that some age-related impairments of the cholinergic system may be due to posttranscriptional events. Immunocytochemistry and retrograde transport labeling results have shown that there is a decline in the number of neurons retrogradely transporting tracers and also, that there is a significant shrinkage in cell surface area in the basal forebrain cholinergic system of aged rats, consistent with there being atrophy of cholinergic basal forebrain neurons and impairment of uptake or retrograde transport mechanisms in the aged brain (34).

2.3. In Vivo Immunolesion Model of a Cholinergic Deficit

In order to understand the mechanisms responsible for age- and AD-associated cholinergic deficits, it is necessary to develop animal models that display specific cholinergic deficits *in vivo* and allow for the evaluation of the biochemical, neuropathological, and behavioral consequences of the lesion model. Models must also be amenable to the study of lesion-induced responses and the repair mechanisms that are triggered by the cholinergic deficits.

At present there is no adequate animal model available which can mimic all of the behavioral, biochemical, and histopathological abnormalities observed in AD patients. However, partial clues can be obtained from a number of cholinergic lesion paradigms that have been used to produce cholinergic hypofunction *in vivo*. These include fimbria-fornix transections, mechanical lesions with radiofrequency and electrolysis, systemic or intracerebral injections of excitotoxins, which are analog of the excitatory amino acid neurotransmitter glutamate (e.g., kainic acid, ibotenic acid, quisqualic acid, NMDA, and AMPA), high-affinity choline transport inhibitors (ethylcholine mustard aziridinium ion, AF64A), or murine anti-AChE monoclonal antibodies. The limitations of these lesion paradigms are that they not only cause cholinergic denervation, but that they also deplete noncholinergic projections, such as GABAergic, serotonergic, noradrenergic, and dopaminergic innervations. Thus, the differential affinities of the different excitotoxins to distinct glutamate receptor subtypes may partly explain the differential cytotoxic effects of each glutamate analogue on different regions of the brain. Selectively destroying cholinergic neurons, although sparing other cell types, would be useful to characterize forebrain cholinergic deficits without the limitations mentioned above (35).

Most of the CBFNs possess NGF receptors in contrast to the other neurons in this region, including cholinergic cells present in the nearby striatum, that do not express detectable levels of NGF receptors (36,37,38). It has been shown that a well-characterized monoclonal antibody to the low-affinity neurotrophin receptor—p75^{NTR}, 192 IgG, accumulates bilaterally and selectively in CBFNs following intracerebral ventri-

cle (i.c.v.) administration. Crosslinking 192 IgG via a disulfide bond to the ribosomal inactivating protein, saporin, generates an immunotoxin (IT), the 192 IgG-saporin complex, that is also taken up via p75^{NTR} receptors and is a specific and selective tool for producing lesions to CBFNs (39). Thus, after an i.c.v. injection of 192 IgG-saporin, the IT is specifically internalized by the terminals of p75^{NTR}-bearing CBFNs, retrogradely transported, and accumulated in the cell bodies of CBFNs. Intraventricular administration of IT produces a selective loss of p75^{NTR}-bearing cholinergic neurons that results in substantial reductions in ChAT activity in a time and dose-dependent manner in the rat basal forebrain and their neocortical and hippocampal afferents (40,41,16,14,42,43,44,45,39,46,47), whereas cholinergic interneurons in the striatum, noncholinergic septal neurons containing parvalbumin (48,14,42) and substantia innominata GABAergic neurons containing calbindin-D28K or NADPH-diaphorase (16) as well as tyrosine hydroxylase-positive neurons (39), glutamic acid dehydrogenase-positive neurons (44), galanin-, neuropeptide Y- and neurotensin B-positive neurons (49,50) are not affected. Not surprisingly, IT also can affect NGF-reactive cerebellar Purkinje neurons at higher doses (16). Prelabeling cortical projecting neurons in NBM with fluoro-gold shows that only those neurons that are also labeled for ChAT are destroyed in the IT-treated animals, suggesting that IT is lethal to cholinergic cells, rather than suppressing cholinergic expression in existing cells (51). Thus, this model allows for dissociation between cholinergic neuronal loss and the attenuation of cholinergic function in spared neurons, a critical distinction if we are to dissect the causes for cholinergic dysfunction in the aged and AD brain.

An early study (52) showed that partial deafferentation of the cholinergic and adrenergic innervations in the hippocampus obtained by destruction of the dorsal routes (through the fimbria-fornix and the supracallosal striae) results in a delayed, but significant recovery of the original cholinergic and adrenergic innervation patterns by remaining cholinergic and adrenergic inputs that reach the target via the ventral route. Because of its slow and protracted time-course and its ability to reestablish innervation in initially denervated areas, this compensatory collateral sprouting phenomenon may be of particular interest for the development of long-term, protracted functional recovery as documented for both experimental brain lesions and accidental severe brain injuries. A partial degeneration to basal forebrain cholinergic projections can also be induced by the administration of sub-maximal doses of IT (53,54,45). These latter partial lesions are comparable to the situation described for AD, in that there is a graded behavioral and biochemical change suitable for the study of cholinergic functional recovery and reorganization in the cholinceptive target areas.

Behavioral studies have shown that using either i.c.v. or parenchymal delivery into NBM or cortex of 192 IgG-saporin (55,56,57,14,58), impairs performance in learning and memory tasks in a manner consistent with the extensive loss of CBFNs (59).

3. TROPHIC ACTIONS OF NGF ON PLASTICITY FOR CHOLINERGIC NEURONS

The neurotrophic hypothesis, that the survival of neurons depends on their competition for neurotrophic factors synthesized in limiting amounts by their target fields, provides the best available explanation for how target fields influence the neuronal

populations that innervate them and the subsequent processes associated with neuronal plasticity (60,61).

3.1. Nerve Growth Factor and Its Receptors

Nerve growth factor (NGF) is the first characterized member of the neurotrophin (NT) family, which also includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and neurotrophin-6 (NT-6) (for reviews, see 62,63,64,65,66,67). NTs are highly basic proteins (pI 9–10.5) each composed of approx 120 amino acids with three intrachain disulfide bonds. Although sharing 50–60% amino acid homology, NTs display distinct, yet overlapping, regional distribution and biological effects that regulate the survival, differentiation and phenotypic maintenance of specific neuronal populations and also play a less well understood role in neuronal plasticity. The biologically active form for NGF is made from a homodimer with two identical beta-subunits. In the central nervous system (CNS), NGF is expressed in different brain areas with the highest levels present in the hippocampus, cerebral cortex, and olfactory bulb, the principal target areas of CBFNs (61,68,69) and affects a variety of cholinergic populations of the forebrain, including those of the medial septum, nucleus basalis of Meynerts, substantia innominata and striatum.

Neurotrophin action is via a receptor-signaling system that is composed of two transmembrane receptor proteins, a p140^{trk} family of the high affinity neurotrophin receptors, and the low affinity neurotrophin receptor, p75^{NTR} (70–73). Most of the NGF receptors in the CNS are synthesized in the CBFNs and anterogradely transported to hippocampal and cortical axon terminals that innervate NGF-producing neurons. NGF interaction with its receptors initiates a series of signal transduction events that begin with the binding of neurotrophins to receptors on the membrane of CBFN terminals in the hippocampus and cortex, internalization of a neurotrophin-receptor complex, and the retrograde transport of the neurotrophin to the neuronal soma.

The p75^{NTR} receptor is a transmembrane glycoprotein which belongs to a family of diverse cell surface proteins that include tumor necrosis factor (TNF) receptors, Fas antigen, and CD40 (74,75). Although p75^{NTR} was originally characterized as the only NGF receptor, it has subsequently been shown to bind the neurotrophins BDNF and NT-3 with a similar K_d ($10^{-9}M$). Immunocytochemical and *in situ* hybridization studies have demonstrated the presence of p75^{NTR} within several distinct populations of neurons in the brains of adult rats and the normal aged human brain, with the highest expression (greater than 95%), of both protein and mRNA, in the ChAT- or AChE-positive cholinergic projection neurons of the basal forebrain, and moderate levels present in cerebellar Purkinje cells, hypothalamus, and brain stem (9,76,77,38).

The p140^{trk} receptor is a *trk*-related proto-oncogene glycoprotein that contains an intracellular tyrosine kinase domain. There are three specific genes forming the *trk* family in mammals, *trkA*, *trkB*, and *trkC*, whose gene products bind NGF, BDNF, and NT-3 respectively (78,79,80). *TrkA* expression is primarily colocalized with p75^{NTR} in CBFNs, although there is no such coexpression in striatal cholinergic interneurons (81,39). Coexpression of the two genes for p75^{NTR} and *trkA* can potentially lead to greater responsiveness to NGF (71) since the p75^{NTR} receptor modulates *trk* receptor function as a result of direct physical interaction between the two receptors (82–84).

The expression of p75^{NTR} and p140^{trk} mRNA in the basal forebrain neurons is also induced by NGF (85).

The p75^{NTR} receptor is involved in the internalization and retrograde transport of all neurotrophins (86). The p75^{NTR} receptor also triggers some cellular responses independent from *trk* receptors by a signal transduction cascade through ceramide that ultimately activates NF-kappaB (87,88) and deters apoptotic cell commitment (89,90,91).

3.1. Neurotrophic Actions on CBFNs

The viability of CBFNs is dependent in part on the neurotrophic support provided by NGF during early development and in adulthood [for reviews, *see* (92,93,94)]. In vitro studies (95,96) have shown that NGF promotes ChAT activity and increases ChAT-immunoreactivity (IR) of neurons exhibiting dendritic and axonal processes as demonstrated using organotypic cultures of fetal rat striatum, although the action of NGF in these cultures may be dependent on ambient conditions (97,98).

NGF effectively prevents the degeneration of axotomized CBFNs in both young and aged animals (99–105). Furthermore, the atrophy of CBFNs and the cognitive deficits displayed by aged rats can be reversed by NGF (106,107). Chronic i.c.v. injections of NGF elevate hippocampal ChAT activity in adult rats after partial septohippocampal lesions (108), induce synaptogenesis and hypertrophy after decortication of adult rats (109), and ameliorate cholinergic neuronal atrophy and behavioral impairment after brain injury or extreme aging (110,109,111).

Hellweg, et al. (112) reported that moderate lesions of the septohippocampal pathway by intraventricular infusions of ethylcholine aziridinium (AF64A) induces a dose-dependent decrease of hippocampal ChAT activity, which returns to control levels within five weeks of treatment. This cholinergic deficit is associated with an increase in hippocampal NGF mRNA, but not BDNF- or NT-3 mRNA. The content of NGF protein transiently increases in the hippocampus before returning to control levels by five weeks after AF64A infusion. At that time, however, NGF content as well as ChAT activity, are significantly increased in the septum, suggesting an increased uptake of NGF by the remaining spared cholinergic neurons. These data provide correlative evidence for a critical role for endogenous NGF in neuroregeneration and plasticity of the cholinergic basal forebrain after incipient damage. Results by Lapchak and Hefti (113) have also shown that chronic or repeated administration of NGF during the onset of degenerative events is necessary for the stimulation of presynaptic cholinergic function in the hippocampus of adult rats with partial fimbrial transections. Another study (114) reveals that cultured basal forebrain from neonatal rats shows both overlapping and nonoverlapping responses to treatment with different neurotrophins. Nonner, et al. (115) demonstrated that NGF and BDNF enhance ChAT activity and survival of cholinergic neurons after hypoglycemic stress. Studies (116,117,118) showed that both NGF and BDNF preserve ChAT activity after lesions, but that only NGF treatment upregulates ChAT or *trkA* expression in individual cells surviving the lesion and induces hypertrophy of the remaining neurons in the lesioned NBM.

Several studies (119,120,54,121) have shown that NGF treatment, by means of NGF infusion or gene transfer, promotes long term CNS neuronal rescue and functional recovery from cognitive impairments associated with aging, lesions of the septohippocampal projection, or partial immunolesions to CBFNs. Dekker, et al. (119) also

reported that grafting of NGF-producing fibroblasts reduces behavioral deficits in rats with lesions of the NBM. NGF-producing fibroblasts, but not beta-galactosidase-producing fibroblasts, ameliorate impairments demonstrated in a Morris water-maze task assay. In addition, spatial acuity is improved to near-normal levels by the NGF-producing graft-treatment. However, ChAT activity in cortical areas and hippocampus is not affected by the NGF-producing grafts. Both experimental and sham grafted groups show a similar reduction in the level of dopamine, but not homovanillic acid or 3-methoxytyramine, in the frontal cortex. Levels of norepinephrine, epinephrine, and serotonin and their metabolites in the neocortex and hippocampus are also not affected by the lesion or the grafts. NGF-producing grafts increase the size of remaining p75^{NTR}-positive neurons in the NBM by 25%. NBM lesions reduce the levels of integrated optic density seen after ChAT-positive fiber staining in the ventral neocortex by 46%, whereas NGF-producing grafts restore staining in this area to 86% of controls. These data suggest that NGF-producing grafts may improve behavior through the partial restoration of the lesioned projections from the NBM to the neocortex.

One study (121) has shown that grafts of substrates favorable axonal growth combined with transient NGF infusions promote morphological and functional recovery in the adult rat brain after lesions of the septohippocampal projection. Long-term septal cholinergic neuronal rescue and partial hippocampal reinnervation are achieved, resulting in partial functional recovery as measured by a simple task that assesses habituation but not a more complex task that assesses spatial reference memory indicating that

- (i) partial recovery from central nervous system injury may be stimulated both by preventing host neuronal losses and by promoting host axonal regrowth and
- (ii) long-term neuronal losses can also be prevented with transient NGF infusions. Others (122,123) have shown that there are long-term protective effects after treatment with human recombinant NGF on primate nucleus basalis cholinergic neurons after neocortical infarction or a unilateral transection of the fornix.

3.2. NGF-mediated Gene Expression

In situ hybridization results (124) relying on use of ³⁵S-labeled antisense oligonucleotide probes show that NGF specifically increases the transcription of the ChAT and m2 muscarinic receptor genes. Chronic infusion of NGF into the forebrain of the adult rat also increases NGF receptor mRNA, ChAT mRNA, and neuronal hypertrophy (125). Since *trkA* mRNA and protein are present in basal forebrain neurons during the entire postnatal period and their distribution is identical to that for ChAT mRNA, *trkA* gene expression also colocalizes to BFCNs (126), where NGF stimulates *trkA* and ChAT mRNA and anti-NGF suppresses the expression of both genes (118). These results suggest that endogenous NGF regulates the expression of *trkA* and ChAT. Finally, whereas NGF infusion increases the size of developing CBFNs, NGF antibody inhibits their normal developmental growth. These results are evidence that endogenous NGF acts on developing CBFNs to enhance gene expression and cellular differentiation. Venero, et al. (127) also reported that intrastriatal NGF infusion after quinolinic acid lesions prevents lesion-induced decreases in ChAT and *trkA* mRNA, but has no effect on glutamate decarboxylase mRNA expression.

Stressful conditions in the CNS, such as ischemia, hypoxia, hemorrhage, trauma, and aging, are known to induce complex responses in neuronal gene expression with

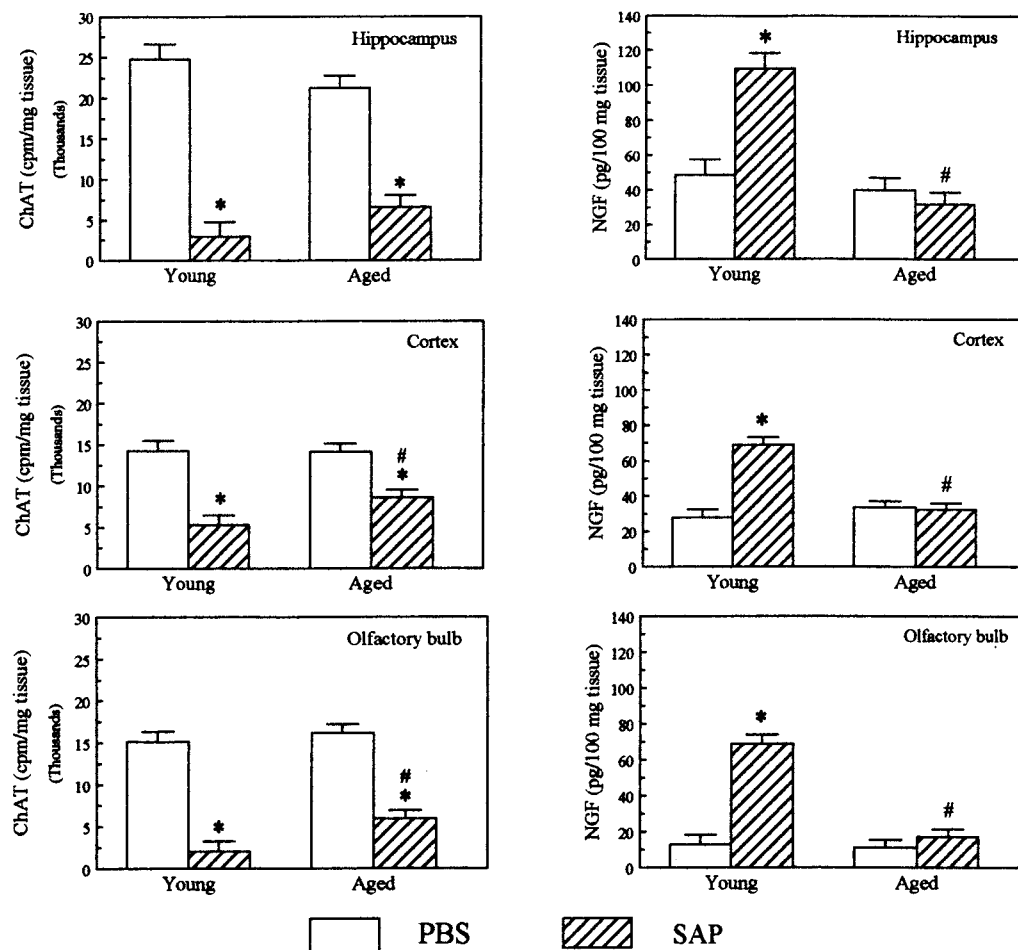


Fig. 2. Effect of total immunolesions on brain ChAT activity and NGF protein levels in the CBFN target areas of young and aged Fisher 344xBrown Norway hybrid rats at 4 week postlesion. Values are mean \pm S.E.M. of 4 young control, 4 young lesioned, 8 aged control, and 7 aged lesioned animals. * $p < 0.05$, significantly different from young control rats; # $p < 0.05$, significantly different from young lesioned rats, two-way ANOVA with post-hoc Fisher's LSD analysis. Adapted from Gu, et al. (131).

delayed effects on neurotransmission and eventual neuronal commitment to apoptosis, two outcome measures with important physiological consequences. There are fewer cholinergic neurons in the aged basal forebrain, and they express lower levels of ChAT and ACh uptake than their counterparts in young rats. These differences are consistent with both age-associated increases in proapoptotic markers and decreases in ChAT activity (3,17,19,34,60,107,128,129,130). Also, cholinergic deafferenting insults to the brain (e.g., fimbria fornix transections or immunolesions) typically result in increases in neurotrophin protein in the brains of young rats, but not their aged counterparts (107,130,131,132) (Fig. 2). These observations are consistent with the hypothesis that there are age-associated impairments of stress response signaling pathways that regulate recovery of neurotransmitter function and abrogate neuronal commitment to apop-

tosis after injury. Although we have information as to the general nature of cholinergic regulation and the more prevalent signals regulating apoptosis in the CNS, the mechanisms that regulate the cholinergic cell loss and the downregulation of ChAT activity in the injured aged or AD brain, the major focus of this project, are less well understood. We hypothesize that the activities of transcription factors that regulate gene expression responsible for neurotransmitter function and apoptotic responses to oxidative stress and trauma are perturbed in the aged brain, in part as a response to chronic stressful events associated with aging-related pathology, some of which is likely to involve oxidative stress. We submit that an understanding of the concerted action of multiple regulatory factors is necessary to understand age-associated changes in gene expression.

4. INTRACELLULAR REGULATION OF CBFNS

4.1. Programmed Cell Death

Programmed cell death (PCD) is a fundamental phenomenon for development and homeostasis maintenance in biological organisms to describe spatially and temporally predictable cell loss. Homeostatic control of cell number results from a dynamic balance between cell proliferation and cell death. It occurs as a physiological process during organogenesis in embryos, as well as in adult cell turnover and differentiation and as a pathological process in response to various injuries [for reviews, see (133,134)]. During the development of the nervous system, neuronal cell death has been considered as a prime example of PCD, which primarily serves to match neuronal number with target innervation. Competition for limiting amounts of neurotrophic factors that are secreted by neuronal targets determines which and how many neurons ultimately survive (135).

Apoptosis, the best characterized form of PCD, is a morphological term coined by Kerr, et al. (136) from ancient Greek for “falling of leaves,” in recognition of its significance in tissue homeostasis. In contrast to necrosis, a term for the features of “accidental” cell death, in which cells undergo swelling, loss of membrane integrity, release of lysosomal contents, and which ends with total cell lysis, apoptosis is characterized by a process beginning with cytoplasmic shrinkage and nuclear condensation, followed by loss of the nuclear membrane, fragmentation of the nuclear chromatin, and subsequent partition of condensed nuclear material and cytoplasm into membrane bound-vesicles defined as apoptotic bodies. These apoptotic bodies are then engulfed by adjacent cells through endocytosis. An inflammatory reaction with infiltration of leukocytes that have typically seen in necrosis is absent.

Apoptosis involves a primary perturbation of nuclear chromatin in which activation or *de novo* synthesis of endonuclease(s) results in cleavage of DNA at linker regions between nucleosomes to form multiple units of chromatin pieces with about 180 base pairs (bp) fragments of double-stranded DNA, which present as a DNA “ladder” by electrophoresis (137,138,139). Appearance of DNA ladders has been reported in many apoptotic processes, and is widely used as a marker of apoptosis with the exception that some necrotic cells also show formation of DNA ladders (140). Furthermore, apoptotic endonucleases also generate free 3'-OH DNA double-strand breaks with the sticky ends, which can be identified using the template-dependent *in situ* end-labeled

translation staining with Klenow DNA polymerase, where the terminal deoxy-transferase dUTP-nick end labeling (TUNEL) detects both sticky and blunt ends (141,142).

Neuronal cell death occurs not only during development but also as a consequence of acute traumatic events or chronic degenerative disorders that disturb cellular homeostasis, such as trauma, ischemia, and aging (143,144,135,136). For example, prolonged ischemia results in varying degrees of neuronal cell death in the region outside the stroke's core, which is affected by a decrease in tissue perfusion that results in an inadequate supply of oxygen, glucose, and other metabolites (137). Although the machinery leading to cell death is not fully understood, some important aspects of the cell death process show the characteristics of apoptosis, such as DNA fragmentation, chromatin condensation, apoptotic bodies, and cycloheximide reduction of ischemic damage (138–145). In addition, studies (146,147,148,149) show that experimental cerebral ischemia induces activation and cleavage of caspase-3 in apoptotic cortex, delayed activation of caspase prolongs ischemic brain protection, elevation of neuronal expression of neuronal apoptosis inhibitory protein (NAIP) reduces ischemic damage in the rat hippocampus.

A wide variety of neurodegenerative diseases such as AD, Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) are characterized by a gradual loss of specific neuronal populations. LeBlanc (149a) reported that in serum-deprived cultured neurons, metabolism of amyloid precursor proteins (APPs) through the non-amyloidogenic secretory pathway is decreased by 20% to 40% in control cultures whereas 4KD amyloid-beta (A-beta) peptide is increased one- to four-fold suggesting that human neurons undergoing apoptosis generate excess A-beta. Zhao, et al. (150) provided evidence that expression of mutant APPs in differentiated PC12 cells induces cell death via an apoptotic pathway. Observations of age-dependent damage, deterioration of respiratory enzyme activities, and susceptibility of mitochondrial DNA to oxidative stress have suggested that the delayed onset and age dependence of neurodegenerative diseases may result from the deterioration of stress response mechanisms (151). A unified model for aging mechanisms encompassing intrinsic mediators and extrinsic agents proposes that altered regulation of gene expression and signal transduction-redox pathways is the basis for age-associated neurodegenerative diseases (152) (Fig. 3). Apoptotic events reflect an interplay between intrinsic signaling events that rely on cytokines, neurotransmitters, and growth factors and responses to extrinsic events that increase levels of reactive oxygen species (ROS). Both intrinsically and extrinsically driven signal-transduction pathways act via transcription factors that regulate the coordinated timely expression of stress-response genes as part of a decision-making process that can commit cells to apoptosis or survival (153).

Neuronal cells, like most other cell types, contain endogenous suicide mechanisms that are activated when specific cell numbers are to be reduced during development, or in response to genotoxic signals (134,154). A report by Sinson, et al. (155) indicates that NGF administration beginning 24 h after fluid-percussion brain injury improves cognitive outcome and decreases cholinergic neuronal cell loss and apoptotic cell death. Another study (156) also shows that NGF prevents cell death in lesioned central cholinergic neurons. NGF also protects hippocampal and cortical neurons against iron-induced degeneration via generation of free radicals (157).

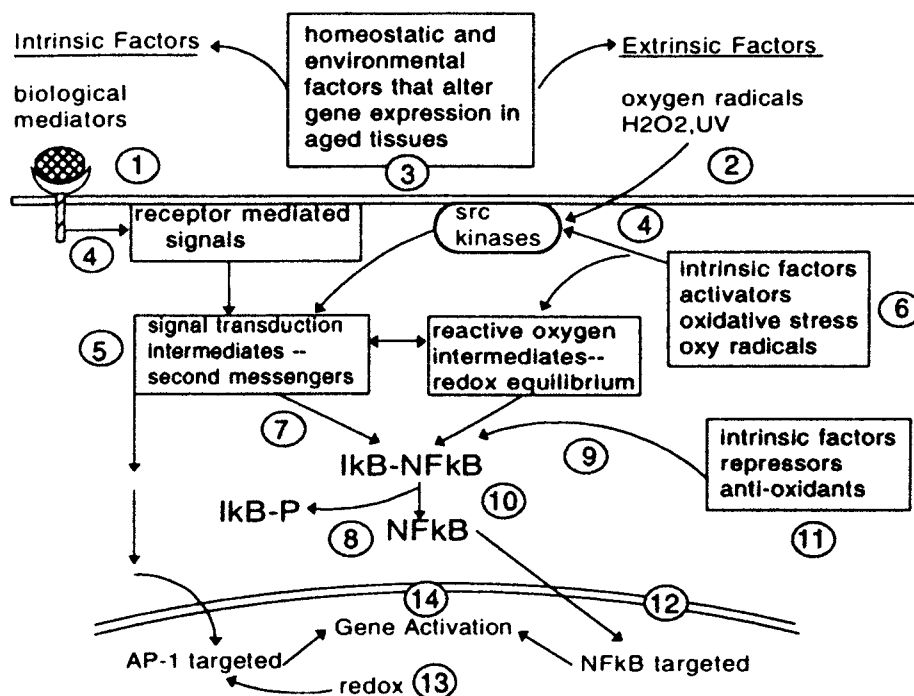


Fig. 3. Model outlining the general interactions of the signal transduction-redox pathways that regulate trans-acting factors of the stress response genes. (1) Biological mediators activate stress response genes via a receptor-ligand complex that transmits signals to the cytoplasm. (2) Extrinsic agents exert their effects upon the early response cytoplasmic intermediate. (3) Homeostatic and environmental changes of intrinsic and/or extrinsic factors could result in altered gene expression and regulation in aged. (4) Membrane-bound *src* kinases are the earliest cytoplasmic factors. (5) Signal transduction intermediates conduct a cascade of modifications that lead to gene regulation. (6) Oxidative metabolism is a source of free radicals that can act as second messengers to stimulate signal transduction intermediates and affect the redox equilibrium. (7–8) Activation of NF- κ B requires its release from I- κ B. (12) Nuclear translocation is mediated by a specific carrier protein. (13–14) Activation of NF- κ B results in gene activation. Modified from Papaconstantinou (152).

Oxidative stress has been linked to neuronal cell death resulting from stressful conditions such as ischemia, trauma, excitotoxicity, and neurodegenerative diseases (158,159,160). As oxidative stimuli, ROS are generated as byproducts of normal and aberrant metabolic processes using molecular oxygen (O₂) as substrate. Cellular defense systems against ROS include enzymes that convert ROS to less reactive species and antioxidants that quench the different ROS directly. Due to the high oxygen consumption and high concentration of oxidizable substances such as catecholamines, ascorbic acid, and lipids in brain, imbalances in oxidative homeostasis result in oxidative stress leading to neuronal damage (161,162). Both transcription factors activator protein-1 (AP-1) and nuclear factor-kappaB (NF-kappaB) play roles in the signal transduction pathways potentially associated with both *trkA*- and *p75^{NTR}*-mediated regulation of apoptosis (163,91,164,165). Serum and NGF deprivation, oxidative stress, and glucose deprivation induce apoptosis in PC12 cells. AP-1 activity

changes are associated with both induced apoptosis and NGF-mediated cell rescue in PC12 cells. The survival of PC12 cells is significantly reduced by the inhibition of NF- κ B activation in the presence or absence of NGF. Apoptosis is induced via the stimulation of several different cell surface receptors in association with caspase activation. Carter, et al. (87) has shown that NGF binding to p75^{NTR} activates the transcription factor NF-kappaB in rat Schwann cells. This activation is not observed in Schwann cells isolated from mice that lacked p75^{NTR}. Selective for NGF, NF- κ B is not activated by BDNF or NT-3.

4.2. Nuclear Factor- κ B

4.2.1. Structure, Regulation and Function

Although the transcription factor NF- κ B was first described as a nuclear factor found in B cells which binds to the immunoglobulin kappa light chain enhancer (166), it is widely expressed outside the immune system. NF- κ B belongs to a family of homo- and hetero-dimeric proteins related by a conserved ~300 amino acid residue NH₂-terminal Rel/homology domain; members of this family include the proteolytically processed p65 (also referred to as RelA), p50 and p49 (also referred to as p52) as well as RelB and c-Rel. The dimeric subunits of NF- κ B can recognize and potentially bind to a 10-bp generic DNA consensus sequence 5'-GGGRNNYYCC-3' (G=guanine, R=purine, N=any nucleotide, Y=pyrimidine, C=cytosine). The most commonly described dimeric subunit combination of NF- κ B is the p65/p50 heterodimer found in the cytoplasm bound to an inhibitory subunit, I- κ B, in the absence of stimulation. Both the p65 and p50 subunits of NF- κ B are regulated by redox modification (166,167,168,169) and manipulations of intracellular glutathione (GSH) and cysteine have significant consequences for NF- κ B activity (170). A role for ROS as second messengers in NF- κ B regulation is suggested by the activation of NF- κ B by H₂O₂ and its inhibition by antioxidants, such as N-acetylcysteine (NAC) and thioredoxin (171,172,173,174,175). Upon stimulation, I- κ B is phosphorylated and degraded (176,177), and nuclear translocation (NLS) domains on NF- κ B proteins are exposed, which facilitate translocation to the nucleus for DNA-binding. Heterodimerization of NF- κ B subunits produces species with various intrinsic DNA-binding specificities, transactivation properties and subcellular localization [for reviews, see (178,179)].

NF- κ B is ubiquitously expressed in most cell types and tissues, although its activation process has been mostly studied in lymphoid cells, epithelial cells, and fibroblasts. NF- κ B is an inducible transcription factor that is responsive to a broad range of stimuli (178,180), such as interleukin-1 (IL-1) and other cytokines, tumor necrosis factor (TNF), bacterial lipopolysaccharide (LPS), human immunodeficiency type-I virus (HIV-1), T-cell mitogens (e.g., lectins, phorbol esters), UVA radiation (181), hydrogen peroxide (182), and hypoxia (183). The converging event for different stimuli appears to be the removal of I- κ B proteins from a cytoplasmic complex with NF- κ B via phosphorylation of I- κ B and subsequent ubiquitination and degradation by proteasomes (184) (185,186). Several kinases, such as protein kinase C (PKC), cAMP-dependent protein kinase and casein kinase II may mediate I- κ B phosphorylation and subsequent dissociation (187). The signal pathway for TNF- α is mediated via the sphingomyelin pathway with the production of ceramide and the activation of PKC ζ (188–193).

Since NF- κ B is a preformed transcriptional factor with regulatory activity, it can be rapidly activated by mechanisms that do not require *de novo* protein synthesis in contrast to the immediate-early transcription factor AP-1 family, whose activity is regulated via prompt, robust and transient gene induction. Activation of NF- κ B by various stimuli leads to subsequent transcriptional activation of many target genes (for reviews, *see 187,7*), including proinflammatory cytokines such as TNF, IL-1, IL-6, interferon- γ ; inducible nitric oxidase synthase (iNOS), manganese superoxide dismutase (Mn-SOD), cyclooxygenase-2 (COX-2); major histocompatibility complex Class-I (MHC-I), vascular cell adhesion molecule-1 (VCAM-1); as well as the neuropeptide dynorphin, and viral HIV-1 gene.

4.2.2. NF- κ B Role in CNS

Although early work was confined to gene activation during the inflammatory and immune response in lymphocytes, epithelial cells and fibroblasts, there is evidence that NF- κ B has a unique role in neurons and astroglia in CNS, where NF- κ B acts in both normal function and acute responses to injury, as well as chronic neurodegenerative disorders—such as AD and PD (for reviews, *see (7,184,187)*).

There is constitutive NF- κ B activity present in the hippocampus, cortex and basal forebrain (*194,195,171,173,196*). In addition to NF- κ B in neuronal cell bodies, both I- κ B- α and NF- κ B immunoreactivity are present in the postsynaptic densities (PSDs) in the rat hippocampus and cortex (*195*). Increased NF- κ B has been identified in neurons and astrocytes of brain sections from AD patients (*197,198*). NF- κ B is also present in the microglia and macrophages in both parenchymal and perivascular areas of multiple sclerosis patients (*199*).

In AD brains, the p65 subunit is most abundant in the particulate fraction of the temporal cortex (*200*) where the proportion of large—probably cholinergic—neurons with nuclear NF- κ B staining is significantly increased (*197,198*). There is increased NF- κ B activity—assayed with an activity-specific monoclonal antibody (mAb) to the p65 subunit—in neurons and astroglia of brain sections from AD patients, where increased NF- κ B activation is restricted to cells in close proximity to early plaques. Also, the neurotoxic peptide A- β is a potent NF- κ B activator in primary neuronal cultures. This activation requires reactive oxygen intermediates as messengers because an antioxidant, pyrrolidine dithiocarbamate, prevents A- β induced NF- κ B activation (*172*). In PD patients, the proportion of dopaminergic neurons with immunoreactive NF- κ B in their nuclei is more than 70-fold higher than that in control subjects (*201,202*). In vitro treatment of primary cultures of rat mesencephalon with C2-ceramide activates the sphingomyelin-dependent signaling pathway resulting in translocation of NF- κ B and the transient production of free radicals that induce apoptosis, indicating a possible relationship between the nuclear localization of NF- κ B in mesencephalic neurons of PD patients and oxidative stress in such neurons.

4.2.3. Delayed NF- κ B Alteration

The outcome of NF- κ B activation may depend on its time course. For example, the transient activation of NF- κ B observed at 24 h after ischemia may be responsible for the induction of protective factors—an antioxidant (LY231617) present in neurons that survive the ischemic insult, whereas the persistent activation of NF- κ B in hippocampal neurons after global ischemia could be responsible for the induction of proteins that

result in CA1 neuronal death (203). Interestingly, this is the opposite of AP-1 where transient increases in activity are associated with apoptosis and persistent increases in activity are associated with neuronal rescue by NGF (165). Thus, NGF treatment of serum-deprived PC12 cells results in a temporally delayed decrease in NF- κ B DNA-binding activity that contrasts with the prompt and robust increases in AP-1 binding activity associated with serum deprivation and are further enhanced and prolonged by NGF treatment. In vivo, transient focal ischemia-reperfusion in the cerebral cortex causes regional alteration of DNA-binding activity of transcription factors AP-1, CREB, Sp-1, and NF- κ B in a time-dependent fashion. There is an increase in AP-1 activity surrounding the ischemic cortex during early reperfusion that is, followed by an increase in the CREB, Sp-1, and NF- κ B, but not AP-1, binding activity in the ischemic cortex itself, and to a lesser extent of Sp-1 and NF- κ B binding activity in the surrounding region, after five ds of reperfusion (204). Traumatic brain injury (TBI) also increases NF- κ B DNA-binding activity in cerebral cortex ipsilateral to the injury site after three days (205). After injection with the toxic glutamate receptor agonist, kainic acid (KA), p65-IR is markedly increased in reactive astrocytes after 7–10 days while binding activity to the kappa light chain enhancer consensus sequence increases two days after KA treatment (206). In spinal cord, there is increased NF- κ B activation that persists for at least 72 h after a contusion injury (207). This injury-induced increase in activated NF- κ B in spinal cord co-localizes with inducible nitric oxide synthase (iNOS) protein, a NF- κ B dependent gene product. These studies suggest that there are critical differences between transient and prolonged activation of transcription factors that determine apoptotic outcomes.

4.2.4. Role of NF- κ B in Cell Death

Results of Bales, et al. (208) show that A- β induced toxicity of cultured fetal rat cortical neurons is associated with internucleosomal DNA fragmentation early after exposure to A- β . There are increased I- κ B- α mRNA and its protein that may be responsible for the retention of NF- κ B in the cytoplasm. In contrast, exposure of rat primary astrocyte cultures to A- β results in the activation of NF- κ B and the subsequent stimulation of IL-1 β and IL-6 levels, suggesting that perturbations in NF- κ B-induced gene expression may contribute to the neurodegenerative and inflammatory response reported for AD. Grilli, et al. (209) have reported that aspirin, and its metabolite sodium salicylate, protect against neurotoxicity in glutamate treated rat primary neuronal cultures and hippocampal slices through inhibition of glutamate-mediated induction of NF- κ B. Ko, et al. (210) has also shown that aspirin exerts its neuroprotective action against NMDA. Also, by blocking the NMDA-induced activation of NF- κ B and JNK. Mattson, et al. (211) have shown that the induction of Mn-SOD by TNF- α and C2-ceramide treatment, and the suppression of peroxynitrite formation and membrane lipid peroxidation by the peroxynitrite scavenger uric acid, is via increased NF- κ B activation. Thus, NF- κ B plays an antiapoptotic role under neurodegenerative conditions resulting from metabolic and oxidative insults due to FeSO₄ and amyloid β -peptide. Furukawa and Mattson (212) have reported that NF- κ B mediates increased calcium currents and decreased NMDA- and AMPA/KA-induced currents in hippocampal neurons treated with TNF- α . Pahan, et al. (213) has shown that the sphingomyelin-ceramide signaling pathway stimulates the expression

of iNOS via LPS- or cytokine-mediated activation of NF- κ B in astrocytes. However, Lezoualc'h, et al. (214) have reported that high constitutive NF- κ B activity mediates resistance to oxidative stress in neuronal cell populations as NF- κ B regulates the transcription of a wide variety of genes that contribute to different physiological outcomes.

To identify whether NF- κ B regulates the transcription of the synthesis of ACh genes and whether this regulation is affected in the aged CNS, it is necessary to assess NF- κ B responses in terms of its binding to the NF- κ B site on the ChAT promoter. The ChAT promoter contains NGF inducible elements and consensus sequences for several transcription factors, including those of the basic helix-loop-helix family (215). The mouse ChAT gene promoter region contains a potential NF- κ B binding site as well as sites that bind the serum response element (SRE), the cAMP response element (CRE), and the activator protein-1 (AP-1) (216,217,218) have shown that nuclear protein extracts from rat basal forebrain and PC12 cultures bind with more affinity to the specific NF- κ B consensus sequence present in the ChAT promoter as compared to the kappa light chain enhancer NF- κ B binding sequence. A supershift reaction using purified NF- κ B proteins has shown that p49 and p65 bind to the ChAT NF- κ B consensus sequence, but not the p50 subunit, in agreement with the hypothesis that different NF- κ B consensus sequences bind to different NF- κ B subunits in a tissue- or cell type-specific manner. Quantitative determination of binding affinities of different NF- κ B proteins to the different consensus sequences (64 different combinations) may provide strategies for selective intervention approaches that may have therapeutic potential.

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Effects of Estrogens and Thyroid Hormone on Development and Aging of Astrocytes and Oligodendrocytes

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1. INTRODUCTION

The regulatory role of hormones and growth factors on glial cell development has been demonstrated in several studies (1–3). Hormones also influence the aging of glial cells and their role in the aging brain (4). In early reports, the gliosis that occurred in the brain of aged animals consisted primarily of astrocytes and oligodendrocytes and was interpreted as a compensatory response to the diminishing structural integrity and functional competence of neurons and their increasing pathology (5,6). The stimulatory effects of thyroid hormones on development and adult function of glial cells are numerous and well established (4,7). Endocrine-glia interactions may support normal function of neurons and repair damage of central nervous system (CNS) regions. The current view is that neurons may regenerate not only during development but also in adulthood, and not only in laboratory animals but also in humans (8–10). The “post-developmental” CNS may not be “as hostile an environment for the regeneration of neuronal networks as once believed” (10) and it may be induced to regenerate by internal influences (8). As glial cells are an important component of the neuronal microenvironment, they may represent a key factor for CNS repair after injury or in prevention and treatment of neurodegenerative diseases.

Glial cells have multiple actions on CNS repair that may be either antagonistic (e.g., formation of a “glial scar” that prevents cell migration to the site of injury) (11,12) or facilitatory (e.g., release of growth factors and axon-guidance and adhesion molecules providing trophic and directional support for regenerating neurons) (12–14). The well-known Spanish neuroscientist Ramon y Cajal had advocated, in 1913, the concept of an “immutable, fixed and rigid CNS” (15). However, Cajal also prophesied that future science may well replace this early concept with that of a more flexible, “plastic” CNS which would respond to environmental and endogenous stimuli capable of modulating neural cells growth, differentiation, and function (15).

Topics in the present chapter focus on the possible role of glial cells as mediators of neural plasticity and the role of hormones thereon. The glial cells studied here

derive from a rat glioma cell line that consists of a mixture of astrocytes and oligodendrocytes, with the proportion of each cell type depending on several factors including the number of cell passages and the type and concentration of the hormone in the culture medium (16–18). C-6 glioma cells provide a useful model to study glial cell properties, glial factors, and sensitivity of glial cells to various substances, including hormones. Among the many hormones/factors significant in glia-endocrine interrelationships, the effects of estrogens and thyroid hormone were studied because of their significance in regulating several important CNS functions, their efficacy in providing reliable treatment of clinical disorders, and the many years they have been investigated in our laboratory.

2. EFFECTS OF ESTROGENS ON PROLIFERATION AND METABOLISM OF GLIAL CELLS

2.1. Estradiol and Tamoxifen Protect Astrocytes and Oligodendrocytes from Glutamate Toxicity

Estrogen actions on neurons have been extensively studied since the 1960s and have been reported to induce maturation, differentiation, and excitation as well as protection of neurons (19–21). By contrast, estrogen actions on glial cells are little and only recently, known (2–4,22–27). In experiments from our laboratory, the ovarian steroid, 17 β -estradiol, and the “designer,” nonsteroidal estrogen, Tamoxifen (triphenylethylene), effectively protected cultured glial cells from the cytotoxicity of the excitatory neurotransmitter, glutamate (28). One of the purposes of these experiments was to compare the effects of 17 β -estradiol, the most biologically potent ovarian steroid, with those of tamoxifen, one of the nonsteroidal estrogens frequently used for treatment (29) and prevention (30) of breast cancer. Traditionally, tamoxifen is considered as an antiestrogen that prevents the actions of estradiol by competitively binding to the two estrogen receptors (ER α β) (23,26,31,32). This competitive binding results in apparently opposite actions, with Tamoxifen being an effective estrogen antagonist in breast tissue but an estrogen agonist in bone, liver, and uterus.

In our experiments, exposure of C-6 glioma cells to 10–20 mM glutamate concentration induced 61–78% cell death. Pretreatment of the cells with 0.01 mM estradiol or with 2 μ M tamoxifen significantly reduced the glutamate-induced cell death, estradiol being the most effective in this regard (Table 1). These estrogen concentrations were selected because previous concentration/effect studies with graded concentrations of estrogens had shown their effectiveness (27,28). Thus, estrogens often used in therapy (estradiol as replacement after menopause and tamoxifen for treatment/prevention of breast cancer) significantly protect glial cells in vitro against glutamate toxicity (28).

Although the mechanism of this protective action may be quite complex, it is known that glutamate is removed from the synapse into astrocytes where it is transformed into the nontoxic glutamine by the enzyme glutamine synthetase abundantly present in astrocytes (33–36). As shown in previous studies (27) and further discussed in subheading 2.2., both estradiol and tamoxifen significantly stimulate glutamine synthetase activity. Thus, one of the mechanisms of neural cell protection against glutamate toxicity by estrogens may be an accelerated conversion of glutamate to glutamine in astrocytes.

Table 1
Comparison of the protective action of 17 β -estradiol and Tamoxifen against glutamate toxicity in glial cells^a

Glutamate concentration mM	% cell death		
	Control without estrogens	17 β -estradiol concentration 0.01 mM	Tamoxifen concentration 2 μ M
10	60.60 \pm 9.98 ^b	2.82 \pm 0.59	18.06 \pm 0.99
15	68.32 \pm 8.92	6.25 \pm 1.15	25.65 \pm 2.13
20	72.75 \pm 10.20	19.95 \pm 0.98	29.67 \pm 1.97

^a C-6 rat glioma 2B-clone cells (50–60 passage cells) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin/fungizone in 95% humidified air with 5% CO₂ and at 37°C. Concentration of 17 β -estradiol (0.01 mM) and tamoxifen (2 μ M) were those proven effective in previous dose-response experiments. Glutamate toxicity was measured by cell counts (with a Coulter Counter ZM) and by the activity of the enzyme lactate dehydrogenase which is released from lysed cells and, therefore, taken as an index of cell death (30,38). Three increasing concentrations of glutamate, 10, 15, and 20 mM, were used.

^b Means \pm SE were analyzed by Student's *t*-test. Cell deaths were statistically ($p \leq 0.05$) higher in controls than in cells treated with both estrogens, and higher in tamoxifen-treated than estradiol-treated cells.

2.2. Estradiol and Tamoxifen may Affect Differently Glial Cell Proliferation and Maturation Depending on Cell Age (Number of Passages) and Type of Estrogen

Estrogens have opposite actions on cell proliferation of early passage cells as compared to late passage cells. In young (10–30 passage) glial cells, 17 β -estradiol promotes cell growth in a concentration-dependent manner (37). However, in studies with older (50–60 passage) cells (27), the response induced by 17 β -estradiol on cell proliferation is inhibitory: the 0.01 mM concentration of 17 β -estradiol that stimulated proliferation in younger cells, reduced cell number by 50% after 8 d in culture in later passage cells; likewise, tamoxifen reduced cell growth in late passage cells, although inhibition was less pronounced than with estradiol (Fig. 1). However, in late passage cells, despite the reduction in cell number, the amount of protein per cell is significantly increased above controls with tamoxifen (by 23%) and even more with estradiol (by 86%) (Table 2). The apparent discrepancy between decreased growth rate and increased protein/cell content suggests that glial cells cultured with estradiol or tamoxifen mature more rapidly than the controls (without estrogen) and thereby become more specialized and lose or reduce their ability to proliferate.

Estradiol stimulates activity of marker enzymes: Glutamine synthetase (GS) for astrocytes and 2'3'-cyclic-nucleotide 3'-phospho-hydrolase (CNP) for oligodendrocytes (involved in the production of myelin). In young glial cells, the increased activity was proportional to the concentration of the hormone. For the 0.01 mM estrogen concentration, GS activity was increased by 65% whereas CNP activity was increased by 20% over controls (37). The stimulation of glutamine synthetase by estradiol is particularly significant in these early passage glial cells; young cultures contain a higher proportion of oligodendrocytes than astrocytes while older cultures contain a higher

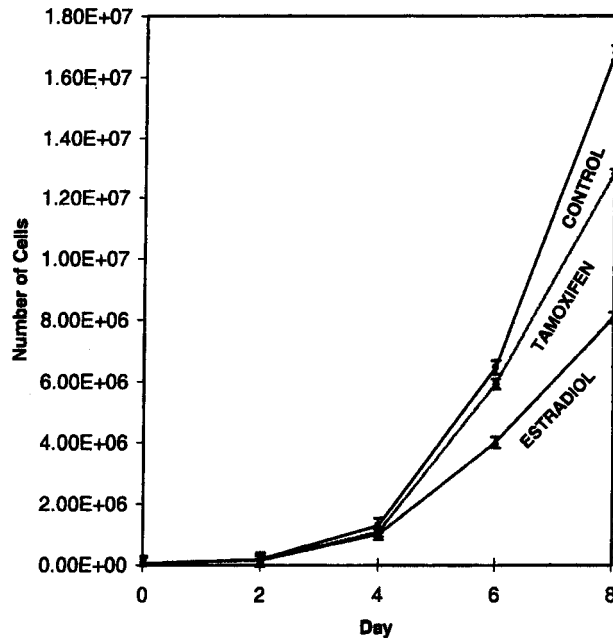


Fig. 1. Comparison of the effects of 17β -estradiol and tamoxifen on proliferation of 50–60 passage glial cells. Cells (C-6 rat glioma 2B-clone, 50–60 passages) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/fungizone in 95% humidified air with 5% CO_2 and at 37°C . Concentration of 17β -estradiol and tamoxifen were 0.01 mM and 2 μM , respectively and were chosen for their effectiveness in previous dose-response experiments. Dots and bracketed lines represent the mean \pm SE.

proportion of astrocytes (16). Therefore, GS activity would be expected to be lower in earlier than in later passage cells where astrocytes predominate. Indeed, in later passage cells cultured in 0.01 mM estradiol concentration, the GS activity undergoes a marked increase over controls of 136% whereas CNP activity is increased by 10% only. Conversely, Tamoxifen significantly increases CNP activity of late passage cells (by 83%) but stimulates GS activity to a much lesser degree than estradiol (Table 2).

With respect to the actions of estrogens on glial cell maturation of late passage cells, histologic examination indicates that both estrogens promote cell maturation as indicated by the more elongated and stellar shape of cell body and the longer and more abundant neurites than in control cells (without estrogens) (Fig. 2). This more advanced maturation is associated with a slower growth but with increased cell protein content and GS and CNP activity.

It may be noted that, whereas estradiol appears to promote astrocytic differentiation, tamoxifen may preferentially stimulate oligodendrocytic differentiation (Fig. 2). The lineage of glial cells, originally thought to be totally independent, may in fact have a common origin. Changes in environmental conditions, such as the addition of specific hormones, may direct the differentiation of glial precursors into cells exhibiting astrocytic or oligodendrocytic phenotypes and, conversely, astrocytes and oligodendrocytes may revert into precursor cells (38). The differential action of the two

Table 2
Comparison of stimulation of total protein/cell and glutamine synthetase (GS) and 2'3' cyclic nucleotide 3' phosphohydrolase (CNP) activity by 17 β -estradiol and tamoxifen in glial cells^a

Estrogen type	% change from control (without estrogens)		
	Protein/cell ^b	GS ^c	CNP ^d
17 β -estradiol 0.01 mM	86.27 \pm 3.31 ^e	136.33 \pm 6.18	9.85 \pm 1.5
Tamoxifen 2 μ M	23.33 \pm 1.55	82.13 \pm 4.17	83.46 \pm 2.87

^a Glial cells (50–60 passages) were grown without (controls) and with estrogens under the same culture conditions described in Table 1.

^b Protein content was measured spectrophotometrically using Coomassie blue dye, by the method of Holbrook and Leaver (1976) *Analyt. Biochem.*, **75**, 634.

^c Glutamine synthetase (GS) activity was measured spectrophotometrically by the method of Rowe et al. (1970) *Meth. Enzymol.*, **17**, 900 and taken as an astrocytic marker.

^d 2'3'-cyclic nucleotide 3-phosphohydrolase (CNP) activity was measured by the method of Prohaska et al. (1973) *Analyt. Biochem.*, **56**, 275 and taken as an oligodendrocytic marker.

^e Means \pm SE were analyzed by Student's *t*-test. Increase in protein/cell was statistically ($p \leq 0.05$) greater after estradiol and tamoxifen treatment than in controls, with estradiol being greater. Enzyme activity was significantly ($p \leq 0.05$) higher for both GS and CNP activity after 17 β -estradiol and tamoxifen treatment. However, GS activity was highest after estradiol and CNP after tamoxifen.

estrogens (27) may correspond to a selective binding of each compound to either of the two ERs and the formation of two distinct, very different estrogen receptor-ligand complexes (39). The different ER-ligand complexes may undergo varying levels of activation, bind to separate DNA-enhancer elements and both functions could lead to differential gene activations. Tamoxifen also has nongenomic, non-ER related actions: It is a potent protein kinase-C inhibitor and at high doses may induce apoptosis (5–15 μ M) (40–42).

The demonstration of the potential ability of tamoxifen to stimulate the differentiation of precursor glial cells into oligodendrocytes, is in agreement with in vivo studies in which estrogens stimulated myelinogenesis in rat brain and spinal cord (43). In demyelinating diseases such as multiple sclerosis, the early active lesions of the disease may undergo an initial stimulation of oligodendrocytic proliferation followed by a decline (44). Estrogens, such as tamoxifen, may stimulate the differentiation of glial cells into oligodendrocytes leading to subsequent remyelination and thereby opening new avenues of therapy for diseases characterized by deficits of myelin formation. Data presented here suggest that the two estrogens studied may influence differentially glial cell maturation: Estradiol would promote the development of the astrocytic expression and tamoxifen would promote the oligodendrocytic phenotype. So far, such a differential maturation is tentatively based on the increased activity of specific marker enzymes (GS or CNP) and cell morphology. Further studies are now ongoing in our laboratory to immunologically identify specific marker proteins to definitely confirm whether each of the two estrogens preferentially stimulates the astrocytic or oligodendrocytic phenotype.

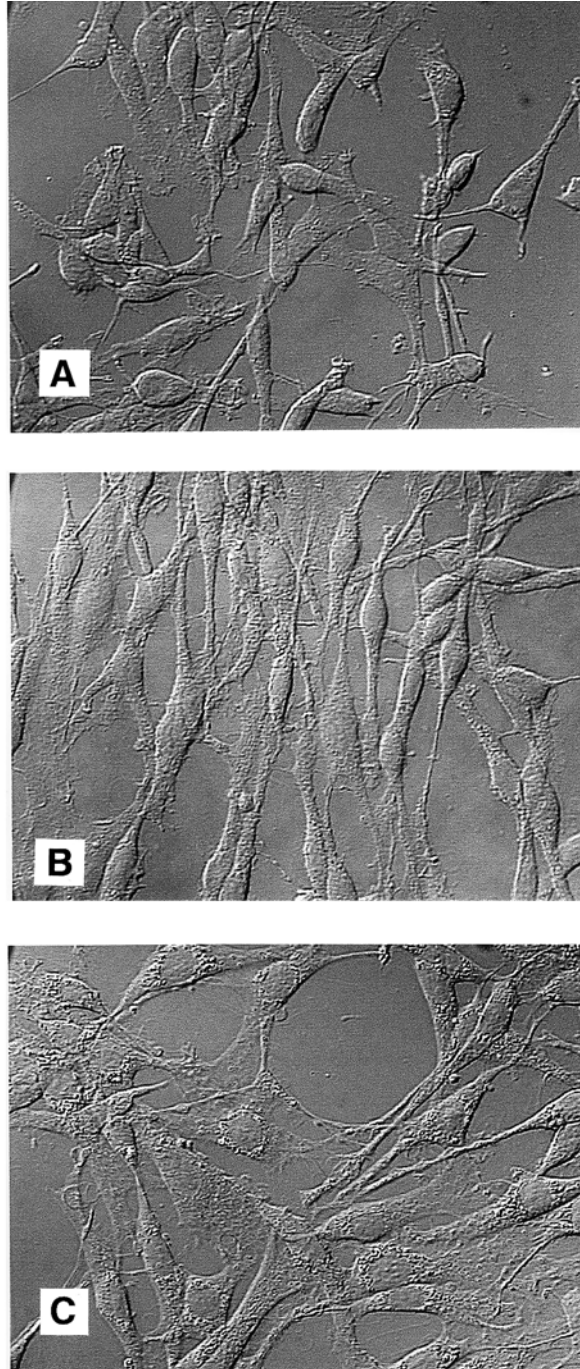


Fig. 2. Estrogens stimulate glial cell differentiation. Cells and experimental conditions were similar to those described in Fig. 1. Cells were grown to 85–90% confluency, washed, trypsinized, resuspended in 10 mL of medium, grown to a concentration of 10^6 mL and then photographed on an Olympus BH2 RFCA inverted microscope with a Technical Instruments COSTAR video camera CV 730 YCB, magnification is $\times 400$ (A) Control cells present a rounded or oblong shape with processes of various lengths. (B) 7-d pretreatment with 17β -estradiol induces maturational changes characterized by more stellate (astrocytic) cell shape, cells oriented in vertical rows, and with longer processes. (C) 7-d pretreatment with tamoxifen also induces maturational changes characterized by a flat, rounded and enlarged cell shape with longer processes forming an extensive network.

3. EFFECTS OF THE THYROID HORMONE, TRIIODOTHYRONINE (T3) ON PROLIFERATION AND METABOLISM OF GLIAL CELLS

The importance of thyroid hormones, particularly the most biologically active hormone, triiodothyronine (T3), in brain development is well documented (4,7). During mammalian CNS development, thyroid hormones are necessary, pre- and neonatally, for cell growth and proliferation of cerebral and cerebellar neurons, proliferation of dendrites, establishment of synapses, synthesis of neurotransmitters, maturation of enzymes for transport and cellular metabolism; during postnatal development, thyroid hormones promote glial cell differentiation, stimulate myelin formation, and local growth factors secretion (e.g., nerve growth factor, NGF). In pre- and neonatal hypothyroidism (i.e., T3 absence or deficit), the severe impairment of brain development results in a profound retardation of neurologic and mental development that leads to "cretinism," a severe form of cognitive deficiency. If, however, replacement therapy with T3 is initiated early during the neonatal period, most of the neurologic and mental damage may be prevented and brain development and maturation essentially proceed as in euthyroid infants. In the adult brain, normal thyroid function is necessary for optimal neurotransmission and for neurologic and behavioral competence: Deficiency or excess of thyroid hormones results in neurologic and mental disorders that may be routinely corrected by replacement therapy (in hypothyroidism) or the use of inhibitors of thyroid hormone biosynthesis (goitrogens) or by surgical or radiological removal of the hypersecreting thyroid tumors.

The presence of T3 receptors has been demonstrated in cultured glial cells (45). These T3 glial receptors, as those on neurons, mediate the major T3 actions both during development and in aging. Although three T3 receptor genes are expressed in several human tissues including the brain, the significance of the expression of multiple genes remains unclear (46). In culture, the number of T3 receptors is significantly greater in neuroblastoma (N2A and N18) than in glioma cells and increases in both cell types when grown in a medium deficient in T3 (45).

3.1. Differential Effects of T3 on Glial Cell Proliferation in Early and Late Passage Glial Cells

In a previous study in young (10 passages) cells cultured with T3, proliferation was stimulated in a concentration-dependent manner: proliferation was unchanged from controls when cells were incubated with the lowest (0.001 mM) T3 concentration; with the two higher concentrations (0.01 and 0.1 mM), proliferation was decreased initially but significantly increased above controls on day 6 in culture, with the highest T3 concentration showing the most growth (47).

Whereas cell proliferation is stimulated in young cells, in old (passage 85) cells, the same T3 concentrations of 0.01 and 0.1 mM inhibited cell proliferation after 6 d in culture, with the highest T3 concentration of 0.1 mM inhibiting cell proliferation the most. Cell proliferation was unaffected by the lowest T3 concentration (0.001 mM) (Fig. 3). As in previous studies, the lowest T3 concentration had no effect on GS and CNP activities, in neither young nor old cells. The intermediate T3 concentration was significantly less effective in the old than the young cells in stimulating both GS and CNP activities (Table 3).

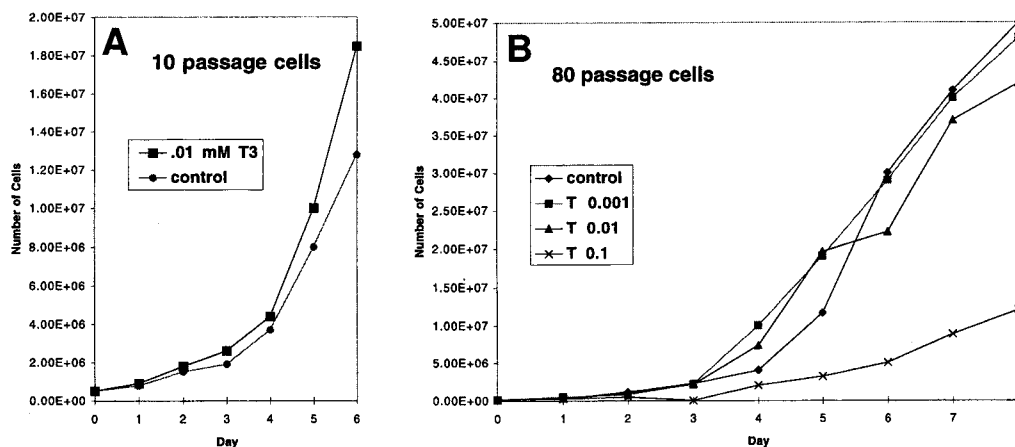


Fig. 3. Comparison of the effects of T3 on glial cell proliferation at early (10) and late (85) cell passages. Cells and culture conditions were similar to those described in Fig. 1. T3 is 3,5,3'-triiodo-L-thyronine.

An overall evaluation of the effects of T3 on GS activity shows that the greatest increase in GS activity (337%) occurs in the old (85 passage) cells with the highest (0.1 mM) T3 concentration. As already mentioned, early passage cultures consist predominantly of oligodendrocytes and fewer astrocytes and the reverse is true for the late passage cultures (16,38). The differential proportion of the two types of glial cells with the number of cell passages is reflected in our findings that stimulation of GS activity by T3 is greatest in late passage cultures rich in astrocytes. The observation of an accelerated maturation of astrocytes after thyroid hormone treatment reported several years ago (1), is supported by the observations presented here, and by other recent studies demonstrating an increased GS expression and activity (3) as well as the increased expression of the glial fibrillary acidic protein (GFAP), another astrocyte marker, in various areas of the brain and in cultured cerebellar astrocytes (48,49).

CNP stimulation is greatest (458%) in the early passage cultures rich in oligodendrocytes (Table 3). The stimulatory action of T3 on oligodendrocytes is in agreement with previous studies that demonstrate a regulatory role of thyroid hormones on myelinogenesis in vivo (50,51).

3.2. Comparison of Estrogen and Thyroid Hormone Actions on Glial Cells: Role of Cell Age and Phenotype and of Hormone Type and Concentration

The observations presented here suggest that estrogens and T3 may influence proliferation, differentiation, and maturation of astrocytes and oligodendrocytes. Which of these actions will be operative at any given time will depend on the hormone itself (estradiol, tamoxifen, or T3) and on the nature (astrocytes vs oligodendrocytes), age (early vs late passage cells), and degree of maturation (immature/progenitor vs mature/differentiated) of the glial cells. Thus, while in early passage cells, both estrogens and T3 stimulate cell proliferation, in late passage cells they inhibit proliferation (Figs. 1 and 2). This age-related differential action on cell proliferation is not unique

Table 3
Comparison of the effects of three T3 concentrations on glutamine synthetase and 2'3' cyclic nucleotide 3' phosphohydrolase activity in young (10 passages) and old (85 passages) glial cells^a

T3 concentration (mM)	% change from control (without T3)	
	Glutamine synthetase	2'3' cyclic nucleotide 3' phosphohydrolase
Young (10 passage) cells		
0.001	1.5 ± 0.04 ^b	10.5 ± 1.6
0.01	171.0 ± 10.6	204 ± 9.8
0.1	277.0 ± 21.5	458 ± 16.5
Old (85 passage) cells		
0.001	4.00 ± 0.23	0.2 ± 0.00
0.01	21.8 ± 0.45	4.6 ± 0.52
0.1	337.8 ± 2.12	244.6 ± 1.82

^a Cells were grown without (controls) and with T3 under the same culture conditions as described in Table 1. Assays for GS and CNP were the same as indicated in Table 2 (c and d). Three graded T3 concentrations were tested.

^b Means ± SE were analyzed by Student's *t*-test. In both young and old cells, enzyme activity was slightly increased above that of controls with the lowest T3 concentration but was significantly ($p \leq 0.05$) higher than than controls with the two higher doses. Peak activity for GS was reached with the highest T3 concentration in old (85 passages) cells and peak activity for CNP was also reached with the highest T3 concentration but in young (10 passages) cells.

to estrogens and T3 but has been demonstrated also in other hormones, such as insulin, and growth factors, such as platelet-derived growth factor (PDGF), each with a specific timetable of action: Insulin and PDGF stimulate growth in late passage cells only (52).

Estrogens and T3 appear to stimulate glial cell maturation overall, as indicated by the increased cell protein content and activity/cell of the marker enzymes, GS for astrocytes and CNP for oligodendrocytes, as compared to controls (cells cultured in medium without the hormones). However, there are some subtle differences in hormone action. Estradiol appears to promote astrocytic expression, whereas tamoxifen seems to selectively promote oligodendrocytic expression (Table 2). T3 stimulation was less effective in stimulating marker enzymes in old (85 passage) cells than in young (10 passage) cells, especially when the hormone concentration was low (Table 3); with the highest T3 concentration, GS activity was greatest in the old passage cells (which are prevalently astrocytic), whereas CNP activity was highest in the early passage cells (which are prevalently oligodendrocytic) (Table 3). In addition to these effects on astrocytes and oligodendrocytes, estrogens may also stimulate microglia to exert both immune stimulatory and antiinflammatory actions and to display neuroprotective actions (53,54). Thus, the availability of a large repertoire of glial responses induced by hor-

mones such as estrogens and T3 reflects the potential significance of hormonal regulation of the neuronal microenvironment.

With increasing progress in CNS rehabilitation, the perspective of restoring neuronal plasticity after damage because of trauma, disease, or aging is becoming a reachable reality. Perhaps, in the adult CNS, inhibition of neuronal replication is the trade-off for neuronal stability necessary for optimal function. However, under appropriate endogenous influences of their microenvironment, neurons may regenerate in animal, and human brains (8,10). Since glial cells, the extracellular matrix, and blood vessels are part of this microenvironment, it may be argued that the study of hormonal influences on glial function represents an indirect but promising approach to eventual therapeutic manipulation of neuronal potential for self-renewal.

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IV

METABOLIC CHANGES

Neurotoxic Injury and Astrocytes

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1. INTRODUCTION

The unique functions of the nervous system are largely attributable to the properties of its electrically excitable cells, the neurons. However, there is a more abundant class of nonexcitable cells, collectively referred to as the neuroglia. Within the central nervous system (CNS) they comprise the astrocytes, oligodendrocytes, and microglia. Progress in the understanding of neuroglial function was originally based upon the pioneering histological staining developed by Golgi and Ramon y Cajal, around 1870 and 1890, respectively. The term neuroglia was derived from the essentially erroneous concept of the German pathologist Virchow (*1*), who postulated that neurons were embedded in a connective tissue to which he coined the name neuroglia, or nerve glue. Although erroneous, it has persisted as the preferred and generic term for these cells, or in its shortened form—"Glia."

Originally, astrocytes were viewed as mere passive support cells for neurons. However, modern experimental techniques have provided ample evidence that astrocytes serve in numerous additional capacities to maintain an optimal environment for neuronal function. Direct contact between astrocytes and neurons determines the morphological and functional differentiation of the latter. It is now well established that the role of astrocytes extends well beyond passive structural support and sensitivity to axon commands. In fact, astrocytes and neurons establish a highly dynamic reciprocal relationship that influences growth, morphology, behavior, and repair within the CNS. Astrocyte interactions with neuronal and nonneuronal cells (oligodendrocytes, microglia, and endothelial cells), and between themselves and the complexity of these interactions provide numerous strategic sites for neurotoxic action. This chapter will provide examples of astrocytic modulation of neurotoxicity. Examples include selective astrocytic toxicants, parent compounds that are metabolized within astrocytes to reactive intermediates with subsequent propensity to selectively damage neurons, as well as toxicants and pathophysiological conditions that affect astrocytic function and lead to altered extracellular fluid composition and secondary neuronal dysfunction.

2. EXCITATORY AMINO ACIDS: THE ASTROCYTIC POOL

Astrocytes occupy about 25% of the brain volume (1A), and their processes are found around synapses and in close association with nodes of Ranvier, axon tracts, and blood vessels. In addition to their structural support for neurons, a partial list of astrocyte functions includes secretion of neurotrophic factors, K⁺ buffering, control of extracellular fluid pH, inactivation of extracellular glutamate, glycogen storage, synaptic remodeling, and uptake and metabolism of neurotransmitters. During development, astrocytes prominently function in guiding neurons to their final target. The physiological functions of astrocytes in the developing and mature CNS extend beyond the scope of this review. Hence, they will not be discussed herein, but the reader is referred to a number of recent publications that provide extensive reviews of astrocytic functions (2–7).

CNS damage in a number of pathological states (e.g., hypoxia, seizures, hypoglycemia, and hepatic encephalopathy), neurodegenerative disorders (Parkinson's disease and Huntington's disease), and aging is thought to be partly due to excessive stimulation of neuronal glutamate-gated ion channels (reviewed in 8). The origin of glutamate (and its analog, aspartate) has been tacitly assumed to be presynaptic nerve endings. However, it is known that astrocytes remove extracellular glutamate by a Na⁺-dependent mechanism (9). This transport has a likely stoichiometry of 1 glutamate and 3 Na⁺ transported inwardly, and 1 K⁺ transported outwardly to offset the negative charge of glutamate. In the presence of ammonia, glutamate is metabolized to glutamine by the astrocyte-specific enzyme glutamine synthetase (GS); (10,11), maintaining [glutamate]_o at 0.3 μM (12,13). This represents a 10,000-fold gradient vs [glutamate]_i (3 mM). This glutamate-glutamine pathway constitutes the pool of brain glutamate originally described by Berl (14). Astrocytes also efficiently remove extracellular taurine by a Na⁺-dependent mechanism (15). This transport system generates and maintains a [taurine]/[taurine]_o of about 10,000, and has a likely stoichiometry of 1 taurine and 2 Na⁺ ions transported inwardly, to generate and maintain the observed taurine gradient (15,16). Release from both the glutamate and taurine pools occurs as a result of astrocytic swelling (2,17). Astrocytic swelling is seen as an early event (within an hour of injury), followed by regulatory volume decrease (RVD). RVD is characterized by astrocyte reestablishment of preswelling volume, a process involving the extrusion of ions such as K⁺ and Cl⁻, and compensatory organic osmolytes (e.g., taurine, myoinositol). To a lesser extent, glutamate and aspartate (17,18) are also released by swollen astrocytes. The prominence of astrocytic swelling in various diseases, the rapidity of the astroglial response and its evolutionary conservation indicate that astrocytic swelling may fulfill a number of important functions. Unlike gliosis it occurs rapidly (19), and may reverse slowly with time. The consequences and the mechanisms of astrocytic swelling are as yet not fully defined, but are beginning to yield to experimental analysis *in vitro*. Astrocytic swelling *in situ* is routinely found to be associated with early pathological states affecting the CNS. Ultrastructural features of head injury suggest that astrocytic swelling precedes neuronal damage (20). A combined magnetic resonance and histochemical study suggests that brain injury after acute cerebral hypoxia is also secondary to astrocytic swelling (21). *In situ*, astrocytes are also known to swell more readily than neurons in response to lactic acidosis and elevated extracellular K⁺, glutamate, and other monoamine transmitters (22,23). Similar findings of

rapid and extensive astrocytic swelling have been reported *in vivo* after cerebral ischemia (23). Mechanistically, astrocytic swelling appears to be a complex phenomenon, with several potential causes and consequences. For example, swelling may occur by simultaneous operation of $\text{Cl}^-/\text{HCO}_3^-$ and Na^+/H^+ exchange transporters, with H^+ and HCO_3^- cycling from the intra- to extracellular spaces via membrane-permeant CO_2 when the increased intracellular NaCl cannot be pumped out. Acidosis occurring with increased lactate increases tissue CO_2 but this will by itself only lead to swelling if $\text{pH}_i \ll \text{pH}_o$, increasing $[\text{HCO}_3^-]_i$ relative to $[\text{HCO}_3^-]_o$. However, when extracellular Na^+ exchanges for intracellular H^+ , and extracellular Cl^- for intracellular HCO_3^- , Na^+ and Cl^- replaces intracellular H^+ and HCO_3^- which can then continue to cycle inside and outside the cell via membrane-permeant CO_2 bringing in one Na^+ and one Cl^- for each turn of the cycle.

Astrocytic swelling and the associated release of excitatory amino acids is likely suspect in mediating neuronal cytotoxicity. In addition, the observed swelling of astrocytes is associated with a close to 50% decrease in the average capillary lumen, likely resulting in decreased blood flow because the flow of red blood cells is impeded. Furthermore, perivascular astrocytic swelling potentially can increase diffusion distances for substrates and waste products to blood vessels that would not otherwise be affected by the primary occlusion.

3. ASTROCYTIC MODULATION OF NEUROTOXICITY

3.1. Methylmercury (MeHg)

MeHg is a particular threat to the CNS, as evidenced by MeHg poisoning in Japan (24), and Iraq (25). Because methylation of inorganic mercury to MeHg by microorganisms is known to take place in waterways (26,27), resulting in its accumulation in the food chain, any source of environmental mercury represents a potential source for MeHg poisoning. Industrial sources (28) of mercury culminating in the acidification of freshwater streams and lakes, and the impoundment of water for large hydroelectric schemes (29) have led to increases in MeHg concentrations in fish, posing increasingly greater risk to human populations.

In the mid 1950s, a chemical plant near Minamata Bay in Japan, discharged mercury into the bay as part of waste sludge. The inorganic mercury was methylated to the organic species, MeHg, and fish and shellfish became contaminated. Consumption of MeHg-adulterated fish by the local population led to an epidemic of MeHg poisoning and severe neurotoxicological and developmental effects (24,30). Following a major drought in Iraq in 1971, the local government opted to switch to a resilient variety of wheat. The order was placed with the government of Mexico. However, a single letter typographical order was made in the name of the fungicide the wheat was to be treated with. Thus, the wheat was treated with MeHg instead of a relatively harmless mercury containing fungicide. The wheat had arrived in Iraq too late to be planted and was used instead to make the traditional pita bread. The farmers were unaware of the significance of the labeling (skull and crossbones poison designation), nor the pink dye additive that was added to warn them of the poisonous nature of the wheat. Weeks later the effects of MeHg intoxication started to appear, leading to mass poisoning epidemic with more than 450 reported deaths (25).

That astrocytes are involved in the etiology of MeHg neurotoxicity is consistent with a number of observations.

1. In vivo, MeHg preferentially accumulates in astrocytes, both in humans and nonhuman primates (31–34).
2. In vitro, MeHg inhibits glutamate uptake in astrocytes (35). Other transport systems examined are 2–5-fold less sensitive to inhibition by MeHg (36).
3. In the absence of extracellular glutamate, cultured neurons are unaffected by acute exposure to mercury (37).
4. Exposure to MeHg is associated with astrocytic swelling in vivo (31,33,34) and in vitro, inhibition of regulatory volume decrease (RVD), as well as increased release of endogenous excitatory amino acids, such as glutamate and aspartate (38).

Several reported actions of MeHg on membrane transporters can lead to astrocytic swelling and release of endogenous glutamate. Based on our, and other investigators' data (39–42) the following model was recently proposed for MeHg-induced astrocytic swelling: MeHg rapidly activates an anion exchange system ($\text{HCO}_3^-/\text{Cl}^-$) leading to unidirectional Na^+ influx and osmotically obligated influx of water. The cellular swelling leads to diminished K^+ clearance from the extracellular fluid either by diminished spatial buffering or reduced KCl uptake, due to the depolarization and appearance of other significant ion conductances, some of which can also lead to a net release of K^+ and Cl^- . Because the astrocytic glutamate carrier is both voltage- and ion gradient-dependent, swelling also reduces glutamate uptake (due to membrane depolarization and increased $[\text{K}^+]_o$). Swollen astrocytes also represent a source for the release of $[\text{glutamate}]_i$ presumably via the activation of leak pathways (38,43). This sequence of events leads to elevated concentrations of excitatory amino acids (EAA) in the extracellular fluid, activating *N*-methyl-D-aspartate (NMDA) receptors and damaging neurons *en masse*.

3.2. Ammonia

Hepatic encephalopathy (HE) or congenital and acquired hyperammonemia result in excessive ammonia (ammonium, NH_4^+) accumulation within the CNS. The condition is due to liver failure. Experimental studies in vivo have shown that the effects of ammonia on the CNS vary with its concentration. At high concentrations of ammonia within the CNS it produced seizures, resulting from its depolarizing action on cell membranes, whereas, at lower concentrations, ammonia produced stupor and coma, consistent with its hyperpolarizing effects.

Ammonia intoxication is commonly associated with astrocytic swelling. In addition, astrocytes undergo morphological changes upon chronic exposure to ammonia, yielding the so-called Alzheimer type II astrocytes common to most hyperammonemic conditions. Notably, the astrocytic changes precede any other morphological change in the CNS (44–46). As alluded to earlier, the exclusive site for the detoxification of glutamate to glutamine occurs within the astrocytes. This process requires ATP-dependent amidation of glutamate to glutamine, a process mediated by the astrocyte-specific enzyme, glutamine synthetase (GS; (47). In vivo chronic exposure to ammonia leads to diminished glutamine metabolism within the astrocytes, as well as impairment of astrocytic energy metabolism (48,49). In addition, it has been reported that the reduced astrocytic capacity to metabolize ammonia leads to ammonia-induced cytotoxicity in

juxtaposed neurons, promoting accumulation of glutamine. The latter, in turn, leads to decreased cerebral glucose consumption and amino acid imbalances (50,51). Increased intracellular ammonia concentrations have also been implicated in the inhibition of neuronal glutamate precursor synthesis, resulting in diminished glutamatergic neurotransmission, changes in neurotransmitter uptake (glutamate), and changes in receptor-mediated metabolic responses of astrocytes to neuronal signals (reviewed in 49). For additional details see chapters 15 and 26 in this volume.

3.3. 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP)

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, or MPTP, is an analog of the opiate analgesic meperidine (Demerol). In 1982, this compound surfaced on the illegal market in the San Francisco Bay area as a “synthetic heroin.” Heroin addicts (generally in their 20s and 30s) exposed to MPTP produced in an unregulated “clandestine” laboratory, rapidly developed a permanent disorder, clinically indistinguishable from nonidiopathic Parkinson’s disease (52). Subsequently, MPTP was shown to specifically damage nigrostriatal dopaminergic neurons. The discovery of MPTP led to resurgence of research on the etiology of Parkinson’s disease, as well as its treatment, for it afforded an experimental model for the disease. When pieced together, the results from many studies suggest a pivotal role for astrocytes in the underlying neurodegenerative changes.

A classical compound that is metabolized within astrocytes to a reactive neurotoxic intermediate is MPTP. The parent compound is metabolized to 1-methyl-4-phenylpyridinium ion (MPP⁺) that selectively destroys nigrostriatal dopaminergic neurons (53–55). The mechanisms of MPTP neurotoxicity, although not fully understood (56,57), suggest that the oxidized pyridinium metabolite (MPP⁺) is the primary mediator of MPTP neurotoxicity. MPP⁺ is apparently able to damage neuronal cells after being formed within and released from astrocytes (57). Because of their ability to accumulate MPP⁺ (via dopamine uptake) and to retain it for a prolonged period of time, dopaminergic neurons are particularly vulnerable to MPTP toxicity. Two pathways of MPP⁺ formation have been identified within astrocytes. The first of these is dependent upon the activity of monoamine oxidase (MAO) and the other is related to the presence of transition metals (57), such as iron. Increased glutamatergic drive to basal ganglia output nuclei has also been postulated to contribute to the pathogenesis of MPTP neurotoxicity. Since astrocytes possess efficient transport mechanisms for both MPTP and glutamate uptake, and increased excitatory tone may be related to aberrant glutamate uptake, recent studies by Hazell et al. (58) have examined the effect of MPTP on astrocytic D-aspartate uptake. Their studies corroborate that MPTP reversibly compromises glutamate uptake in cultured astrocytes, and that this effect is dependent on the conversion of MPTP to MPP⁺. Such findings suggest that the glutamate transporter in astrocytes may play an important role in MPTP-induced neurotoxicity (58). Another recent study investigated the cellular changes within astroglial cells in the MPTP-lesioned striatum. Specifically, striatal expression and regulation of connexin-43 (cx43), the principal gap junction protein of astroglial cells were evaluated (59). These studies confirm that MPTP is cytotoxic altering the expression and protein levels of astrocytic cx43, this providing for another possible mechanism for MPTP-induced neurotoxicity.

That astrocytes mediate an essential step in MPTP toxicity is also supported by *in vivo* studies with the astroglial-selective toxicant, α -aminoadipic acid (α -AA; *see also*

below) (60). When MPTP is directly injected into the substantia nigra, a loss of nigral neurons is observed as revealed by fluorescent retrograde axonal tracing. In contrast, co-injection of MPTP plus α -AA into the substantia nigra is associated with reduced neuronal degeneration, presumably due to the initial destruction of resident astrocytes, and reduced conversion of MPTP to MPP⁺. The protective effect of α -AA is curtailed, however, if reactive astrocytosis is allowed to proceed and MPTP is injected into the substantia nigra one week after the initial injection of α -AA. Thus, once repopulated with astrocytes (astrocytic scar), MPTP is again oxidized to MPP⁺, leading to nigral damage and loss of dopaminergic neurons. Furthermore, because of the increased number of astrocytes upon the formation of an astrocytic scar (gliosis), MPTP-damage is increased compared to the experimental paradigm where it is co-injected with α -AA, reflecting increased metabolic conversion of MPTP to MPP⁺. It is noteworthy, however, that other researchers were unable to reproduce the gliotoxic effects of α -AA (61). Although the two groups used very different methods to assess the degree of cell death, the reason for the different outcomes of these studies remains unclear. Clearly, these studies point out the importance of convincingly deciphering the modality and permanence of the effects of α -AA within the context of astrocytic functions, and provide added impetus for future studies to outline the mechanistic link between MPTP and its cytotoxicity.

3.4. Methionine Sulfoximine

Methionine sulfoximine (MSO) is an irreversible inhibitor of the astrocyte-specific enzyme, GS (62). This enzyme in the presence of ammonia catalyzes the conversion of glutamate to glutamine. When administered to animals, MSO leads to rapid convulsions (see below). Although at present, there appears to be no known environmental exposure to MSO, the literature is replete with examples of accidental poisonings with this compound. For example, wheat flour that had been bleached with agene (containing nitrogen trichloride) and accidentally consumed by dogs led to "running fits", "canine hysteria", convulsions, and anoxia (63–65). Oxidation of methionine residues during the bleaching process of the wheat proteins is believed to have led to the formation of the toxic species, MSO.

At the morphological level, ingestion of large amounts of adulterated flour resulted in neuronal cell loss in the hippocampal fascia dentata and pyramidal cell layer, in the short association fibers and lower layers of the cerebral cortex, and in cerebellar Purkinje cells. MSO leads to large increases of glycogen levels (66), primarily within astrocytic cell bodies, but not in other neuroglial cells (oligodendrocytes and microglia) or neurons (67). In astrocytic cell bodies and in subpial, pericapillary and perineuronal astrocyte processes the glycogen often completely fills the cytoplasm, crowding the remaining organelles and inclusions. MSO treatment is also associated with swollen and damaged astrocytic mitochondria (68). An ultrastructural study of cerebral cortex following administration of MSO revealed morphologic changes in astrocytes, consisting of cytoplasmic enlargement, mitochondrial and rough endoplasmic reticulum proliferation, development of cisternal and saccular smooth endoplasmic reticulum, nuclear chromatin clumping, and hydropic degenerative changes (69,70). As noted by these authors, these changes are similar to those seen in experimental ammonia encephalopathy, suggesting perhaps an important role of ammonia in the evolution of

these morphologic changes. Prolonged exposure to MSO was recently shown to lead to the appearance of swollen astrocytes, with watery nuclei reminiscent of Alzheimer type II glia, primarily in the neocortex, hippocampus, and lateral thalamus (71). In agreement with earlier studies (66), these changes were accompanied by simultaneous accumulation of glycogen in the superficial three layers of the neocortex, hippocampus, and pyriform cortex. GS immunoreactivity appeared enhanced in the cortex, hippocampus and lateral thalamus with a parallel increase in GFAP immunoreactivity. It is noteworthy, that the area of glycogen accumulation coincided with the known distribution of *N*-methyl-D-aspartate (NMDA) and glutamate receptors (71). This suggests that GS may play an important role in NMDA receptor-mediated glutamate metabolism.

Although it is generally accepted that MSO inhibits GS, it remains unclear whether this inhibition represents the primary mechanism of MSO neurotoxicity. Studies in cultured astrocytes incubated in the presence of [³H]guanosine and MSO have shown that three of the four [³H]methyl guanines formed were more highly labeled in the [³H]tRNA of the MSO-exposed cells, relative to that of the control cells (72). These findings suggest a stimulatory effect of MSO on the methylation of neural tRNA guanines, which was previously observed both in vitro using [¹⁴C]S-adenosyl-L-methionine and in vivo using [methyl-³H]L-methionine. These authors suggest that the effect of MSO is mediated via an effect on methyltransferase enzymes (73). The same group has also reported that following intraperitoneal administration of MSO, S-adenosyl-L-methionine levels in brain are maximally reduced at the same time that MSO reaches its highest CNS concentration. Unfortunately, however, a mechanistic link between an effect on methyltransferase enzymes and cellular morphology and specificity was not established.

The relationship between inhibition of GS by MSO and seizure generation is also not well understood. Recent hypotheses have focused on the role of glutamate and GABA in seizure generation, since glutamine provides the precursor for these neurotransmitters. Rothstein and Tabakoff (74,75) have demonstrated that the calcium-dependent, potassium-stimulated release of glutamate and aspartate was inhibited in striatal tissue after intracerebroventricular injection of MSO. Addition of glutamine to the perfusion medium could reverse this effect. Furthermore, MSO has been shown to reduce the synthesis and stimulation-induced release of GABA and glutamate both in brain slices and in vivo, pointing to the importance of glutamine synthesis for neurotransmitter amino acid synthesis (76,77). In other studies MSO increased the rate of efflux of newly loaded radiolabeled glutamine from rat cortical astrocytes in primary culture to more than 400% of the basal efflux, but it did not affect the efflux of the neurotransmitter amino acids, GABA or D-aspartate, under the same experimental conditions. It was suggested by these authors, that MSO-induced overflow with glutamine, which strongly interacts with the NMDA receptor complex, may therefore contribute to the convulsive action of MSO.

3.5. α -Amino adipic Acid (α -AA)

A six-carbon homologue of the excitatory amino acids (EAA), glutamate and aspartate, α -amino adipic acid (α -AA) is naturally produced in small amounts within the CNS as an intermediate of the degradation of L-lysine (78). An inborn error of metabolism where the oxidative degradation of α -keto adipic acid is depressed (produced by

the transamination of α -AA) is associated with abnormally large urinary excretion of α -AA. These same individuals also have increased plasma levels of α -AA (79,80).

Toxicity associated with the racemic mixture of α -AA (D,L- α -AA) was first uncovered in the mouse model (81). D,L- α -AA was shown to affect ependymal and glial cells in the hippocampal arcuate nucleus, as well as Muller cells of the retina (81). Additional studies have shown that enantiomorph type (D- vs L-), the temporal profile of exposure, as well as the tissue or cell type involved, determine the cytotoxic outcome. Other factors are also known to determine cell susceptibility to α -AA or its racemic mixture (D,L- α -AA). For example, when D,L- α -AA is applied to C6 neurogliomas they readily degenerate. However, if the cells are pretreated with dibutyryl cyclic AMP or sodium butyrate prior to D,L- α -AA exposure the cells appear insensitive to the racemic mixture (82).

As alluded to in the section on MPTP neurotoxicity the ability of α -AA to selectively destroy astrocytes is somewhat controversial. The precise reason for different outcomes of astrocytic degeneration upon microinjection of α -AA in different experimental approaches remains unclear. The mechanism by which α -AA exerts its cytotoxicity is also a subject for debate. It is generally accepted that this effect is not attributable to activation of the EAA receptors, because specific ligands of these receptors (NMDA, kainate, AMPA) do not induce gliotoxicity (83–86). The uptake of α -AA was reported to be astrocyte-specific, and it was purported to be Na⁺-dependent and insensitive to tetrodotoxin (87,88). A number of potential mechanisms for the astrocyte-specific effects of α -AA were postulated. These include:

1. a reduction of intracellular glutathione levels and subsequent oxidative damage (82,89) and
2. a rapid increase in the transient of intracellular Ca²⁺ (90). In the absence of mechanistic understanding on α -AA neurotoxicity, it is rather surprising that there are no published papers on the effect of α -AA in astrocytes since 1993.

3.6. Fluoroacetate And Fluorocitrate

The Krebs cycle inhibitor fluorocitrate (FC) and its precursor fluoroacetate (FA) are taken up in brain preferentially by glia. FA occurs naturally in a number of plants in the southern hemisphere, and is available commercially as a rodenticide (Compound 1080). It is prevalent in the South African plant *Dichapetalum cymosum*, commonly referred to as the Gifblaar plant. Exposure to FA may also occur via exposure to the anticancer drug 5-fluorouracil (91). Ingestion of large amounts of FA results in ionic convulsions within 30–60 min. Animals consuming FA commonly seize within minutes of exposure, and those surviving these episodes frequently die later on due to respiratory arrest or heart failure. Ruminants are particularly sensitive to FA, likely because during digestion in the prestomach they rely on formed acetate as an energy source (86).

The actions of FC and FA have been attributed to both the disruption of carbon flux through the Krebs cycle and to impairment of ATP production (92). FA can be metabolized to fluoroacetyl CoA, followed by condensation with oxaloacetate to form FC by citrate synthase (93). A second hypothesis implies that FA toxicity is associated with the inhibition of a bi-directional citrate carrier in mitochondrial membranes (94), which would also be expected to lead to elevated intramitochondrial citrate and could

affect citrate-dependent ATP synthesis (95). Finally, it has been suggested that elevated citrate, secondary to inhibition of aconitase, is associated with the cytotoxicity of these compounds. The latter catalyzes the reactions involved in mitochondrial energy production.

Both FA and FC have been shown to reduce the incorporation of radioactive label from several substrates into glutamine to a much greater extent than into glutamate (96). Since glutamine synthesis occurs exclusively in astrocytes, it was suggested that the reduction of glutamine labeling is related to a direct inhibitory effect of FA and FC on GS. However, this does not appear to be the case. A predominant theory attributes the reduction in astrocytic glutamine to impaired uptake by astrocytes of neuronally released glutamate by FA (97). Other studies have demonstrated that FA applied by microdialysis acted locally on astrocytes and, therefore, impaired astrocytic function was hypothesized to contribute to the development of hepatic encephalopathy by facilitating the entry of ammonia into the brain (98). Inhibition of excitatory synaptic transmission by elevated brain ammonia has been suggested by the same authors as a potential mechanism for CNS depression in hepatic encephalopathy. Other findings strongly suggest that endogenous citrate released specifically from astrocytes into the extracellular space in the brain may function to modulate NMDA receptor activity (99).

These findings, as well as those by Hassel et al. (100) collectively suggest that FA lowers the level of glutamine, and inhibits glutamine formation in the brain *in vivo*. Furthermore, this occurs not by depletion of glial cells ATP, but by causing a rerouting of 2-oxoglutarate from glutamine synthesis into the TCA cycle during inhibition of aconitase (100). After the inhibition of aconitase, citrate accumulates, whereas the levels of isocitrate, and α -ketoglutarate decrease. The reversible enzyme glutamate dehydrogenase begins to work in the opposite direction feeding more α -ketoglutarate into the TCA cycle (86).

3.7. Potential Astrocytic Involvement in Neurodegenerative Disorders

Mucke and Eddleston (101), Eddleston and Mucke (102), Benveniste (103), and Norenberg (104) recently reviewed astrocyte roles in CNS immune responses. The prevailing theories about CNS immune responses suggest that astrocytes, in concert with microglial cells recruit and activate infiltrating hematogenous cells and that they regulate blood-CNS interfaces. Reactive astrocytes produce and secrete a host of cytokines, proteases, protease inhibitors, adhesion molecules, and extracellular matrix components all of which mediate immune and inflammatory responses. Astrocytes have been previously implicated as the cells that are responsible for the initiation of inflammatory demyelinating disease by presenting CNS antigens to autoreactive immune cells. However, more recent studies discount such a role for astrocytes, and favor resident microglia as the CNS antigen presenting cells (APC) (reviewed in (102,103)).

In reviewing the role of glial cytokines in CNS repair, Mrak et al. (105), have identified the following cascade as potentially playing an etiologic role in Alzheimer's disease. The microglial immune response-generated cytokine, interleukin-1 (IL-1), upregulates both the expression and processing of β -amyloid precursor proteins (β -APPs), hence favoring β -amyloid deposition, the morphological hallmark of Alzheimer's disease. Proliferation and activation of astrocytes are also promoted by β -APP, in turn, up regulating S100 β protein synthesis and secretion by astrocytes.

Increased concentrations of extracellular S100 β increase the intracellular concentrations of Ca^{2+} within neurons, stimulating neurite growth and neuritic plaque formation. Direct β -amyloid activation and/or Ca^{2+} -induced neuronal injury and death further stimulate microglial release of IL-1, an added stimulus for astrocytic activation. Accordingly, chronic, self-propagating, cytokine-mediated molecular and cellular reactions are invoked to explain the progressive neurodegeneration and dementia of Alzheimer's disease (105). A similar mechanism has been postulated in the etiology of temporal lobe epilepsy (106) and HIV (107). Interestingly, both CNS IL-1, as well as S100 β immunoreactivity are reported to be increased in Alzheimer's disease (108). In temporal lobe neocortical tissue surgically resected from patients with intractable epilepsy, astrocytic S100 β -immunoreactivity is also 3–5 times higher than in control patients (106). Stanley et al. (107) have recently noted an increase in the number of activated astroglia expressing elevated levels of S100 β in HIV-infected patients. Although at this point these findings are viewed as correlative, an assessment of the relative contribution of astrocytes to neurodegenerative disorders should be a particularly fruitful subject for future studies.

Granulocyte/macrophage-colony stimulating factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF) are cytokines necessary for growth and differentiation of macrophages. Both cytokines have been reported to lead to an accumulation of macrophages at the site of inflammatory lesions. Furthermore, both GM-CSF and G-CSF enhance a number of functional activities of mature macrophages, such as their phagocytic, cytotoxic, and microbicidal activities (reviewed in (103)). GM-CSF and G-CSF produced locally by astrocytes are an essential element for the recruitment and activation of hematogenous cells. Secretion of G-CSF and GM-CSF by astrocytes would, therefore, be expected to increase granulocyte and macrophage survival within the CNS and augment their activity against invading microbes. However, because viral replication in cultured HIV-infected monocytes is increased by GM-CSF, it has been suggested that astrocytic release of GM-CSF may augment viral production in monocytes and microglia, thus potentially worsening the spread of the infection within the CNS (101,109). That astrocyte cytokine production, namely transforming growth factor- β (TGF β), may lead to the recruitment and spread of cell-borne virus in HIV-1 infection was also postulated by Wahl et al. (110). However, as suggested by Eddleston and Mucke "many cytokines appear to fulfill a multitude of functions and their effects in the intact adult CNS are only now beginning to be defined. It is therefore, perhaps not too surprising that the effects of cytokines in specific neurologic diseases have been difficult to predict." Exposure of primary cultured rat astrocytes to the major HIV envelop glycoprotein, gp120, and also leads to alterations of ion and solute transport (111). It is yet to be explained how these changes contribute to neuronal cell injury associated with AIDS dementia.

4. SUMMARY

The foregoing Subheadings have provided a brief discussion on the potential involvement of astrocytes in neurotoxicity. Both in vivo and in vitro studies corroborate that, in principle, astrocytes are capable of damaging neurons in response to chemical exposure. Both direct impairment of astrocyte function by parent compounds or metabolism of a parent compound to an intermediate metabolite can play an active role in

assaulting healthy neurons of the CNS. The relatively sparse amount of information currently available on the effects of neurotoxic compounds on astrocytes makes the expansion of such studies timely and worthwhile. Expanded investigations on astrocytic involvement in neurotoxicity is clearly warranted, and as new experimental tools are developed it is likely that further strides will be made in understanding astrocytic-mediated mechanisms of neurotoxicity and neurodegeneration.

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Ammonium Ion Transport in Astrocytes

Functional Implications

Neville Brookes

1. INTRODUCTION

1.1. Brain-to-Blood Distribution of Ammonia

The gross compartmentation of ammonia in the brain appears as if consistent with the commonly held assumption that the uncharged base (NH_3) is much more permeant than its conjugate cation (NH_4^+). For example, the normal brain-to-blood concentration ratio of ammonia is in the range 1.5–3 (1), where “ammonia” is defined as the total of NH_3 plus NH_4^+ . This is the predicted distribution when the concentrations of NH_3 in blood and brain are nearly equal, and the differences in NH_4^+ levels are determined simply by the pH of each compartment and the pK_a of ammonia, which is approx 9.2 in plasma. An estimated intracellular pH of 7.1 in brain, as compared with 7.4 in blood, accounts for a twofold excess of NH_4^+ in the brain. This distribution of NH_4^+ approximates the distribution of total ammonia because more than 98% of ammonia exists as NH_4^+ at pH 7.4 or below.

1.2. Effect of Ammonia on Intracellular pH

The rapid intracellular alkalization usually observed when neural cells are exposed to ammonium chloride is further evidence of the relative permeability of cell membranes to NH_3 (2). Alkalinization occurs because NH_3 , entering more rapidly than NH_4^+ , associates with cytosolic H^+ . Boron and DeWeer (2) observed that the NH_4Cl -induced alkalization of squid giant axon was followed by a slowly acidifying “plateau phase” (similar to the methylamine tracing shown in Fig. 1). They hypothesized that a slow influx of NH_4^+ contributes to this gradual decline of pH_i toward baseline. Once a steady-state distribution of NH_3 is reached, entering NH_4^+ now dissociates to form H^+ and NH_3 , the latter diffusing out of the axon. Ammonium ions enter because an electrochemical gradient for NH_4^+ entry continues to exist even when the intracellular and extracellular concentrations of NH_3 equalize at steady-state (3).

The basis for this continuing inward electrochemical gradient of NH_4^+ is as follows. Assuming that the value of $\text{pK}_a (= [\text{H}^+][\text{B}]/[\text{BH}^+])$ is not different inside the cell than

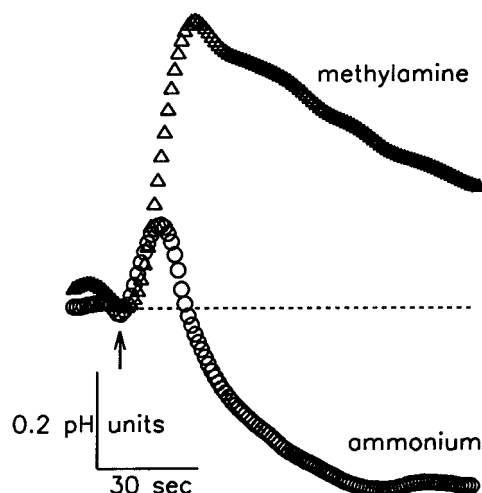


Fig. 1. Effect of ammonium (O) contrasted with methylamine (Δ) on intracellular pH (pH_i) in mouse cerebral astrocytes. At the arrow, each weak base (10 mM) was added in nominally bicarbonate-free solution at pH 7.4. Upward deflection of the superimposed fluorometric recordings indicates alkalinization. The dashed horizontal indicates resting pH_i (~ 7.1). Recordings are smoothed and replotted from Fig. 2 of (4).

outside, it may be stated that when $[\text{B}]_i = [\text{B}]_o$, then $[\text{H}^+]_i/[\text{H}^+]_o = [\text{BH}^+]_i/[\text{BH}^+]_o$ (where B is base, BH^+ is conjugate cation, and subscripts i and o indicate inside or outside of the cell). Stated in other terms, the equilibrium potential for NH_4^+ adopts the same value as the equilibrium potential for H^+ . Because active extrusion of acid by pH-regulatory transport makes the H^+ equilibrium potential considerably less negative than the membrane potential in most cells, thus creating an electrochemical gradient for H^+ influx, a similar gradient will drive NH_4^+ entry. Boron and DeWeer (2) found that their quantitative model of the response of intracellular pH (pH_i) to NH_4Cl best fit their data when the permeability of the membrane to NH_4^+ was 10^5 times lower than the permeability to NH_3 (that is, $P_{\text{B}}/P_{\text{BH}^+} = 10^5$)

1.3. Reevaluating Ammonia Fluxes in Astrocytes

An effect of NH_4Cl on pH_i resembling that described by Boron and DeWeer (2) has been observed in a variety of neurons and glia. However, more recent findings in glia indicate that the initial alkalinization may be absent or greatly curtailed, whereas the subsequent acidification seen in the continued presence of NH_4Cl is greatly intensified (4–6). This behavior suggests rapid transmembrane fluxes of NH_4^+ , and small $P_{\text{B}}/P_{\text{BH}^+}$ ratios of less than 10^3 . It is argued here that these low values of $P_{\text{B}}/P_{\text{BH}^+}$ are likely to be associated with the accumulation of ammonia in astrocytes. Existing evidence is compatible with a role for astrocytic accumulation of ammonia. For example, an overall brain-to-blood ratio of 3 allows for the possibility of severalfold regional variation in ratios among different intracellular compartments. When blood ammonia increases, the brain-to-blood ratio is observed to rise transiently to ~ 8 or more (1).

The purpose of this chapter is to summarize the evidence that fluxes of NH_4^+ across the cell membranes of some mammalian astrocytes are more rapid than previously suspected, and to explore functional implications relating to the distribution of ammonia in the brain. Because NH_4^+ fluxes are primarily via transport pathways shared by K^+ , and neuronal activity raises extracellular K^+ concentration ($[\text{K}^+]_o$) (7), it is predictable that the interaction between NH_4^+ and K^+ fluxes will be activity-dependent. A “potassium-ammonium countercurrent” model is proposed that hypothesizes opposing fluxes through the astrocytic syncytium, with NH_4^+ migrating toward, as K^+ migrates away from, regions of increased neuronal activity (4,8). The reactive astrocytes observed to proliferate in the aging brain, and in neurodegenerative disease, express an altered K^+ -transport phenotype (9) that could disrupt this hypothesized potassium-ammonium countercurrent.

2. AMMONIUM ION TRANSPORT AND DISTRIBUTION

2.1. Mammalian Astrocytes

Figure 1 illustrates the markedly differing effects of methylamine hydrochloride and NH_4Cl on pH_i in mouse astrocyte cultures (4). Methylamine hydrochloride elicits the classic response to a permeant weak base with a relatively impermeant conjugate cation. In this astrocytic response, the decline of pH_i during the plateau phase following methylamine-induced alkalinization is likely to involve pH -regulatory transport (for example, $\text{Cl}^-/\text{HCO}_3^-$ exchange) and metabolic production of acid (10). By contrast, NH_4Cl elicits a brief alkalinization succeeded rapidly by a marked and sustained acidification below resting pH_i . Figure 2 shows that the initial velocity of this acidification, expressed in terms of NH_4^+ influx, does not approach saturation in the concentration range 0.25–20 mM NH_4Cl , suggestive of channel-mediated NH_4^+ flux.

Low concentrations of Ba^{2+} inhibited up to 81% of the acidifying NH_4^+ influx produced by 1 mM NH_4Cl (see Fig. 3), implicating inwardly rectifying potassium (Kir) channels as the major path for NH_4^+ entry. Kir channels are the predominant K^+ channels determining the membrane potential of astrocytes, and they are characteristically sensitive to Ba^{2+} (11,12). Although the initial velocity of acidification did not saturate, the steady-state acidification did so at concentrations above 5 mM NH_4Cl . Steady-state acidification saturates because as pH_i falls, the velocity of pH -regulatory acid extrusion increases and the electrochemical gradient driving NH_4^+ influx declines, limiting the maximum extent of acidification.

Astrocytes also transport NH_4^+ actively (4,6). Bumetanide (100 μM) blocked 34% of the acidifying influx elicited by 1 mM NH_4Cl , indicating that NH_4^+ is taken up via cation-chloride cotransport (13). In glia acutely isolated from the retina of the bee, cation-chloride cotransport of NH_4^+ accounted entirely for an acidifying response to NH_4Cl (6). However, in mouse astrocytes, the combination of Ba^{2+} plus bumetanide was required to prevent net acidification in response to 1 mM NH_4Cl (see Fig. 3).

A well-established precedent for rapid, acidifying uptake of NH_4^+ by active and passive routes exists in the thick ascending limb of Henle's loop (14). In this nephron segment, the primary routes of NH_4^+ flux across the apical membrane similarly are Ba^{2+} -inhibitible K^+ channels and bumetanide-inhibitible $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporters carrying NH_4^+ in place of K^+ . It is noteworthy that the estimated value of $P_{\text{B}}/P_{\text{BH}^+}$ in

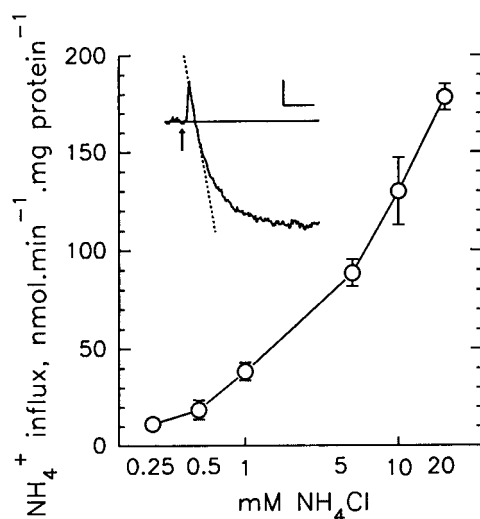


Fig. 2. The initial velocity of intracellular acidification (expressed here as equivalent influx of NH_4^+) in mouse cerebral astrocytes does not saturate with increasing NH_4Cl concentration. The plot was generated from data (means \pm SEM, $n = 3-6$) shown in Fig. 3B of (4), using estimates of intracellular buffering power ($15.8 \text{ mmol.l}^{-1}.\text{pH unit}^{-1}$) and intracellular volume ($4 \mu\text{L/mg protein}$) (23). **Inset:** Typical response of pH_i to $5 \text{ mM NH}_4\text{Cl}$ (added at the arrow) in nominally bicarbonate-free solution (calibration, 0.1 pH u/30 s). The initial velocity of intracellular acidification is given by the slope of the dashed line.

rabbit thick ascending limb was as low as 20 (15). In other words, the apical membrane may be only 20-fold more permeable to NH_3 than to NH_4^+ .

2.2. Role of Inwardly-Rectifying Potassium Channels

The cloned inwardly rectifying K^+ channel Kir4.1 is expressed predominantly in mammalian glia (16). The astrocytic property of high membrane K^+ conductance, resulting in a membrane potential close to the K^+ equilibrium potential, is likely to be in some measure a function of Kir4.1 expression (17). Consequently, Kir4.1 channels, which are blocked by $3-30 \mu\text{M Ba}^{2+}$ (16), are also a probable route of Ba^{2+} sensitive, passive NH_4^+ entry in astrocytes. The findings on the regulation and modulation of Kir4.1 channels suggest a basis for the variable permeability of glia to NH_4^+ , and point to limitations of astrocyte culture as a model system. Expression of Kir4.1 disappeared when glia isolated from mammalian retina were maintained in culture for 4 d (17). By use of the appropriate attachment factor and hormone supplementation during culture, either diffuse or clustered Kir4.1 expression could be restored to the cell membrane. Moreover, coexpression of specific membrane-associated anchoring proteins triggered both the clustering of Kir4.1 and marked enhancement of the Kir4.1-mediated current (18).

Functional studies in astrocyte cultures show that Kir conductance is more evident in stellate, process-bearing cells than in morphologically-undifferentiated, proliferating astrocytes (12,19). It is well documented that K^+ fluxes in astrocytes cultured from mouse cerebrum exceed those measured in rat astrocytes cultured under similar conditions by at least 10-fold, suggesting that the expression of Kir conductance, in cultured astrocytes also varies with species (20). Thus the evidence strongly suggests that high

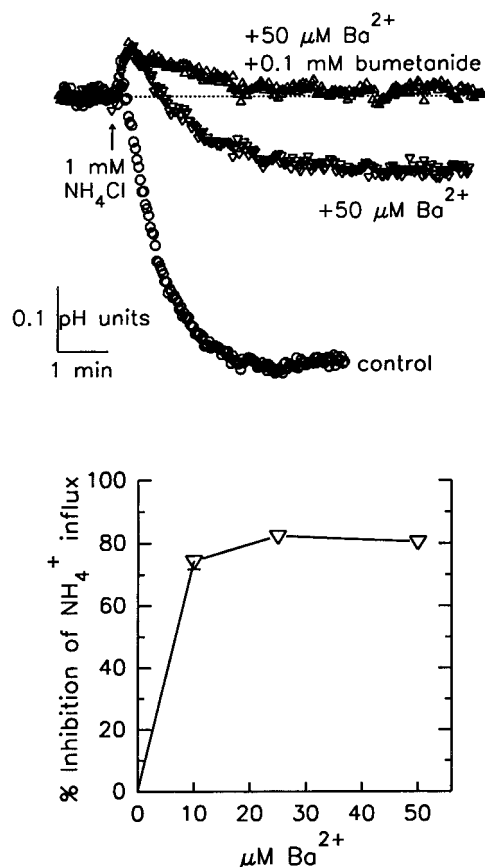


Fig. 3. Ammonium-induced intracellular acidification in mouse cerebral astrocytes is blocked by inhibitors of Kir channels (10–50 $\mu\text{M Ba}^{2+}$) and cation-chloride cotransport (0.1 mM bumetanide). **Top:** Cumulative inhibition by Ba^{2+} and bumetanide. Recordings of fluorometric responses to 1 mM NH_4Cl in the presence and absence of inhibitors are shown superimposed on a dashed horizontal indicating resting pH_i . **Bottom:** Inhibition of initial acidification rate, expressed as NH_4^+ influx elicited by 1 mM NH_4Cl , saturates at low $[\text{Ba}^{2+}]$ (means \pm SEM, $n = 7\text{--}11$). The data are replotted from Figs. 7A and 6B of (4).

Kir conductance, together with an associated high permeability to NH_4^+ , is a feature of the postmitotic astrocyte phenotype whose expression is variably subject to impairment in cell culture. It is reasonable to conclude that the use of astrocyte cultures can lead to an underestimate of the extent of NH_4^+ permeation in vivo. This is not to imply uniformly high Kir conductance in astrocytes in vivo. There is clear evidence of nonuniform distribution of Kir conductance not only within the cell membranes of single glia (21), but also between regionally distinct glial populations (22).

2.3. Effects of Extracellular Potassium

The extracellular potassium concentration in the brain is basally 2–3 mM , rising maximally to ~ 12 mM in areas of intense neuronal activity, or higher when neural

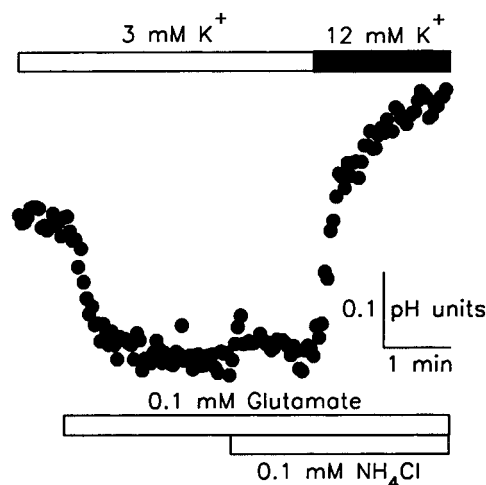


Fig. 4. Dominance of K^+ -induced intracellular alkalization in mouse cerebral astrocytes exposed to 0.1 mM glutamate plus 0.1 mM ammonium, under conditions of physiologic bicarbonate buffering (26 mM HCO_3^- , gassed with 5% $CO_2/95\% O_2$). The fluorometric recording is replotted from Fig. 2A of (31).

depolarization is pathologically sustained (7). Astrocyte pH_i , extracellular pH, and NH_4^+ transport are all subject to regulation by $[K^+]_o$ (4,23,24). Thus $[K^+]_o$ is a key variable affecting the distribution of ammonia in astrocytes.

2.3.1. Potassium-Dependence of Intracellular pH in Astrocytes

The sodium-bicarbonate symport is a pH-regulatory transporter found characteristically in glia, and not detected so far in neurons (10). Inwardly transported HCO_3^- increases pH_i by associating with H^+ . Carbonic anhydrase activity catalyzes the conversion of carbonic acid thus formed to membrane permeant CO_2 and water. Transport of each Na^+ ion is coupled to the transport of either 2 or 3 HCO_3^- ions, resulting in a net movement of charge and a consequent dependence of transport on membrane potential (25). Potassium-induced depolarization of the astrocyte membrane accelerates influx of HCO_3^- via the symport, thus alkalizing the cytosol, the loss of HCO_3^- to the intracellular compartment causes extracellular carbonic acid to dissociate and acidify extracellular pH (24).

Potassium- and ammonia-induced pH changes are affected very differently by physiologic bicarbonate buffering. Because the effect of $[K^+]_o$ on pH_i is mediated by HCO_3^- transport, this effect is fully expressed in physiologic bicarbonate buffer (~26 mM HCO_3^- , see Fig. 4) and markedly diminished in HCO_3^- -depleted solution (23). In a sense, $[K^+]_o$ regulates the “set-point” at which pH-regulatory transport seeks to maintain pH_i . By contrast, the direct effects of NH_3 and NH_4^+ on pH_i are attenuated by bicarbonate buffering, as well as by the intrinsic buffering capacity of the cytosol and by pH-regulatory transport (4). Normal extracellular levels of ammonia (0.1–0.2 mM) show little effect on astrocyte pH_i when applied in bicarbonate-buffered solution in vitro (see Fig. 4).

It is noteworthy that K^+ -induced alkalization is the dominant effect of neuronal stimulation in vivo on astrocyte pH_i (26), surmounting the acidifying action of glutamate (see

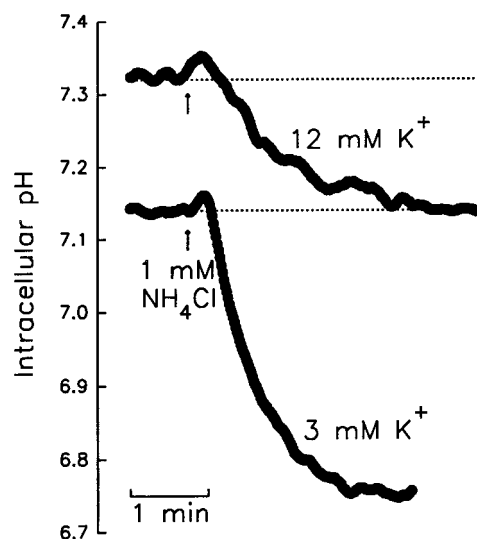


Fig. 5. Potassium inhibits ammonium-induced intracellular acidification in mouse cerebral astrocytes. The fluorometric recordings, replotted from Fig. 5D of (4), show responses of pH_i to 1 mM NH_4Cl in nominally bicarbonate-free solution containing 3 mM or 12 mM K^+ . The upper recording is displaced by the alkalinizing effect of increased $[K^+]_o$.

Fig. 4) and other mediators and metabolites released by neuronal activity. A large physiologic increase in $[K^+]_o$ from 3 mM to 12 mM in vivo can reverse the transmembrane gradient of pH in astrocytes. This would eliminate the normal concentration gradient of NH_4^+ between the astrocyte compartment and blood, assuming that P_B/P_{BH^+} is high. However, it will be argued here that the effect of $[K^+]_o$ on ammonia distribution is amplified further when P_B/P_{BH^+} is basally low and rises with increasing $[K^+]_o$.

2.3.2. Effects of Potassium on Ammonium Transport

Studies in mouse astrocyte cultures show that increasing $[K^+]_o$ in the physiological range markedly slows the acidifying influx of NH_4^+ (see Fig. 5). A depolarization-induced decline in the inward electrochemical gradient of NH_4^+ is unlikely to account for this effect of K^+ because it is offset by an increase in this electrochemical gradient caused by K^+ -induced alkalinization of pH_i . Further, this inhibitory effect of K^+ on NH_4^+ -induced acidification is too large to be attributable to reduced cation-chloride cotransport of NH_4^+ (4). The remaining possibility is that K^+ inhibits permeation of NH_4^+ via Kir channels. However, raising $[K^+]_o$ is well known to increase the K^+ conductance of Kir channels in astrocytes (12). Thus inhibition of NH_4^+ permeation presumably involves some form of competition between K^+ and NH_4^+ for transport via these channels (27).

2.4. Dependence of Ammonium Ion Distribution on Permeability

When NH_3 is the permeating species and NH_4^+ is assumed relatively impermeant, the distribution of ammonia is independent of cell membrane potential and is dependent only on the pH of the intracellular and extracellular compartments, as outlined in the Introduction. The opposite condition of very low P_B/P_{BH^+} results in a distribution

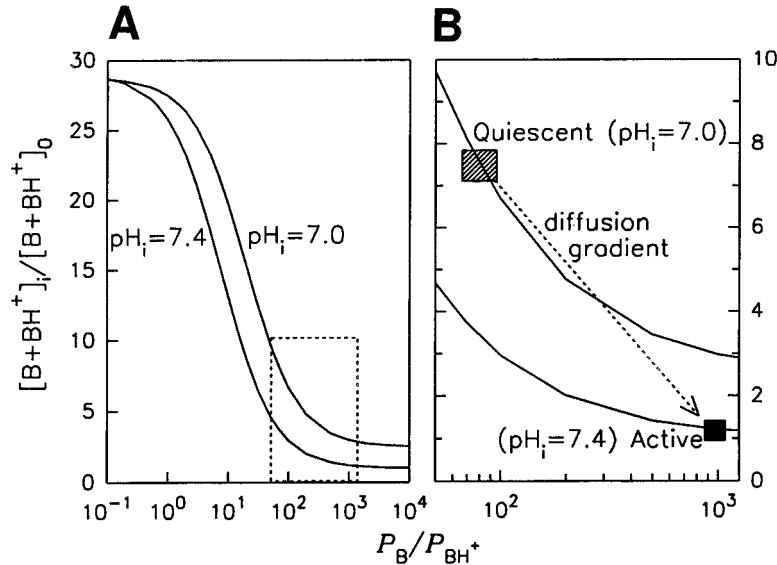


Fig. 6. (A) Distribution ratio of total ammonia ($B + BH^+$) as a function of the relative permeability of astrocyte membrane to the conjugate species (P_B/P_{BH^+}), plotted using the following expression (Eqn. 3 of Ref. 29):

$$\frac{[H^+]_i + K_a}{[H^+]_o + K_a} \cdot \frac{(P_B/P_{BH^+}) - \{f[H^+]_o/K_a(1 - e^f)\}}{(P_B/P_{BH^+}) - \{f[H^+]_i e^{>f}/K_a(1 - e^f)\}}$$

where $f = FV_m/RT$ (V_m is membrane potential, and F , R and T have their usual meaning). Plots were constructed for the putative limiting physiologic values of astrocyte pH_i shown ($pH_o = 7.4$, $pK_a = 9.2$, $V_m = -90$ mV, and temperature = 37°C). (B) An expansion of the area of Graph A enclosed by the dashed line. See **Subheadings 2.4.** and **3.2.** of the text for discussion.

of the conjugate cation determined entirely by the membrane potential, and independent of pH. Roos and Boron (28,29) explored quantitatively how the distribution of weak bases relates to intermediate values of P_B/P_{BH^+} between these two extremes. The expression developed by Roos (28) is used in figure 6 to model the transmembrane distribution of total ammonia in astrocytes as a function of P_B/P_{BH^+} . This relationship is plotted for two different values of pH_i , representing approximate limits of pH_i variation in response to the physiologic range of $[K^+]_o$. Significant voltage-dependent intracellular accumulation of NH_4^+ is seen when the value of P_B/P_{BH^+} falls below 10^3 . Observations of a rapid acidifying influx of NH_4^+ in astrocytes justify considering the functional implications of P_B/P_{BH^+} values in this low range.

3. POTASSIUM-AMMONIUM COUNTERCURRENT

3.1. Astrocyte Regulation of Extracellular Potassium

The maintenance of neuronal excitability by buffering $[K^+]_o$ is a glial function that has been explored extensively (11). The expression of K_{ir} conductance, and perhaps $K_{ir4.1}$ channels specifically, in astrocytes is thought to be critical for this function (17).

Proposed mechanisms of K^+ homeostasis rely on a high-conductance pathway for K^+ influx which remains open throughout the physiologic range of $[K^+]_o$. The “spatial buffering” model proposes that K^+ enters astrocytes in regions where neuronal activity raises $[K^+]_o$, and exits at a distance in electrically more quiescent regions of lower $[K^+]_o$. A second proposal is that astrocytes transiently accumulate K^+ , together with chloride and water, when $[K^+]_o$ rises in electrically active regions. Both mechanisms create an intracellular concentration gradient of K^+ within astrocytes, or within a syncytium of astrocytes coupled by gap junctions, such that K^+ flows away from active zones towards more quiescent zones. The following consideration of the effect of $[K^+]_o$ on ammonia distribution leads to the conclusion that an opposing gradient of intracellular NH_4^+ concentration exists, causing NH_4 to migrate away from quiescent zones towards active zones.

3.2. An Intracellular Gradient of Ammonium Concentration

As already discussed, pH_i and P_B/P_{BH^+} are major factors governing the intracellular concentration of NH_4^+ . In astrocytes, the evidence suggests that both of these factors are regulated by $[K^+]_o$. A localized elevation of $[K^+]_o$ will elicit a localized depolarization-induced alkalization when the space constant of the astrocyte cell membrane is small compared to the dimensions of the cell or syncytium. Given the physiological range of $[K^+]_o$ in the brain, reasonable limits of pH_i are 7.4 in an alkalized active zone and 7.0 in a quiescent zone. From the curves shown in figure 6, assuming for the moment that P_B/P_{BH^+} is high ($>10^3$) throughout the membrane, these values of pH_i translate into an approximately threefold concentration gradient of intracellular NH_4^+ , causing NH_4^+ to migrate *toward* the active zone. Note that the curves in figure 6 are based on a constant extracellular pH of 7.4, whereas variations in $[K^+]_o$ regulate extracellular pH as well as pH_i . Thus it is the distribution of ammonia between astrocytic cytosol and blood that is modeled here, rather than the distribution between astrocytic cytosol and the extracellular compartment of the brain.

When the evidence of rapid NH_4^+ permeation in astrocytes is taken into account, allowing that P_B/P_{BH^+} may fall below 10^3 , figure 6 shows how the intracellular gradient of NH_4^+ concentration is further amplified. Because the apparent permeability of NH_4^+ declines in the presence of increased $[K^+]_o$, it follows that P_B/P_{BH^+} is higher in the active zone. However, in the absence of direct measurements, the range of values of P_B/P_{BH^+} selected in figure 6B represents no more than a partially informed guess. It should be noted that the time course of K^+ -induced effects on pH_i and P_B/P_{BH^+} differ markedly. Responses of pH_i to changes in $[K^+]_o$ are slow, with a half-time of ~ 0.5 min in vitro (23), whereas direct effects of $[K^+]_o$ on P_B/P_{BH^+} presumably are very rapid.

The dependence on pH of the velocity of ammonia metabolism by glutamine synthase is an additional factor that potentially can amplify the intracellular gradient of NH_4^+ concentration, and speed the migration of NH_4^+ toward an active zone. Glutamine synthesis from ammonia and glutamate is maximal near the pH optimum for glutamine synthase activity, which coincides with an active zone pH_i of 7.4 (30,31).

Figure 7 is an attempt to summarize in a simple scheme some of the factors that generate a potassium-ammonium countercurrent within a syncytium of astrocytes. The arguments supporting this scheme suggest that normal extracellular ammonia concen-

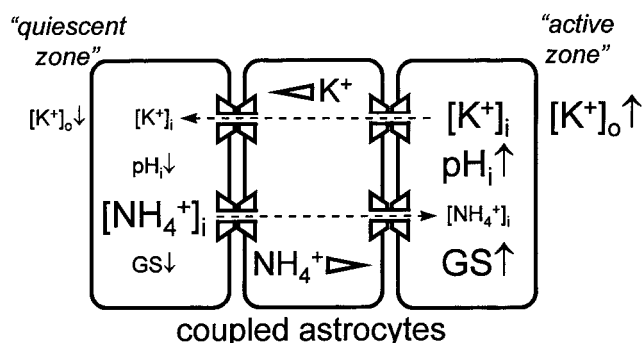


Fig. 7. Hypothetical intracellular "potassium-ammonium countercurrent" in a glial syncytium, between neurally active and neurally quiescent zones. In the active zone, $[K^+]_i$, pH_i and glutamine synthase activity (GS) increase, and $[NH_4^+]_i$ decreases, in response to rising $[K^+]_o$. Opposite changes occur in the quiescent zone. See **Subheading 3.2.** of the text for discussion.

trations of 0.1–0.2 mM (1) result in intracellular NH_4^+ concentrations of 1 mM or more in astrocyte quiescent zones, and 0.1–0.2 mM or below in active zones. Assuming that quiescent zones are normally more extensive than active zones, the intracellular flux of NH_4^+ produced by this gradient could balance much or all of the opposing flux of K^+ away from active zones. It should be noted that the short space constant characteristic of high-conductance glial cell membranes favors this scheme, although presenting difficulties for the spatial buffering model (11). Nevertheless, a potassium-ammonium countercurrent presumably would complement other mechanisms of K^+ homeostasis rather than represent an alternative to them.

4. HYPERAMMONEMIA AND AMMONIUM ION TRANSPORT

Measurements of astrocyte pH_i and ammonia distribution in animal models of human hyperammonemia appear at first sight to challenge the interpretations of in vitro data offered here, but further consideration reveals a basis for consistency. Specifically, two studies of hyperammonemic rats report *elevations* of glial pH_i in vivo relative to controls (32,33), in contrast to the acidifying responses of astrocytes in vitro. However, high ammonia concentrations cause a loss of intracellular K^+ that raises $[K^+]_o$ markedly in vivo (34,35), whereas the volume bathing astrocyte cultures is too large for $[K^+]_o$ to be affected by such K^+ loss. As noted in **Subheading 2.3.1.**, K^+ -induced alkalization is a dominant regulator of pH_i in vivo. For example, the recordings in Fig. 5 show that, even in a nominally bicarbonate-free solution, raising $[K^+]_o$ from 3 mM to 12 mM alkalizes pH_i and diminishes the acidifying effect of 1 mM NH_4Cl sufficiently that steady-state pH_i in the presence of 1 mM NH_4Cl does not differ from control pH_i at $[K^+]_o = 3$ mM. In physiologically bicarbonate-buffered solution, the K^+ -induced alkalization is amplified, and the NH_4^+ -induced acidification reduced by >70% (4), such that the combined response of pH_i to 1 mM NH_4Cl and elevated $[K^+]_o$ will be net alkalization in vitro as in vivo. Interestingly, 1 mM NH_4Cl *acidified* pH_i in brain slices (36), perhaps because the short diffusion path to the bathing solution diminishes the ability of K^+ to accumulate extracellularly in the slice.

Similar arguments can be invoked to account for the transience of the rise in the brain-to-blood ratio to ~8 or more observed after an increase in blood ammonia concentration (1). Ammonia-induced membrane depolarization and loss of intracellular K^+ occurs slowly, in a time frame of tens of minutes (37). This allows astrocytes to accumulate ammonia initially, with a subsequent decline in distribution ratio induces pH_i as rising $[K^+]_o$ to alkalinize and P_B/P_{BH^+} to increase. However, these factors are not sufficient to account for the inability of brain slices to accumulate ammonia when exposed to ≥ 1 mM NH_4Cl in vitro (38). The evidence suggests that these high concentrations of NH_4Cl only partially depolarize astrocytes (37), have little effect on astrocyte Kir conductance (34,37), and acidify pH_i in brain slices (36). Some other, as yet undetermined, mechanism must prevent ammonia accumulation under these conditions in vitro.

A related question is why, if the brain-to-blood ratio can rise transiently to ~8 at the onset of hyperammonemia, is a similarly high brain-to-blood ratio not found at normal blood ammonia concentrations which do not cause a loss of intracellular K^+ ? A possible reason is suggested by the observation that methionine sulfoximine, an inhibitor of glutamine synthase activity, increases the normal ratio by approximately threefold (1). Ammonia accumulation in astrocytes may therefore be limited normally by a velocity of ammonia fixation exceeding NH_4^+ influx. Similar reasoning suggests that diminished glutamine synthase activity in vitro may contribute to the large distribution ratio of ~19 measured in brain slices when the ammonia concentration in the bathing solution is not raised above the normal physiologic range for blood (38).

5. REACTIVE ASTROCYTES

Glial proliferation occurs at loci of neuronal degeneration in the normal aging brain, also early in the formation of the senile plaques of Alzheimer's disease, and commonly in other neurodegenerative disorders (39). The membrane properties of proliferating "reactive astrocytes" revert to a phenotype expressed during gliogenesis, and evident also in a variety of other proliferating cell types (9). There is a marked decrease in Kir conductance and an increase in outwardly rectifying K^+ conductance. This signature change is detectable in gliotic tissue of patients with recurrent seizures, as well as in the proliferating astrocytes of a cell culture model of gliosis (9). Given the key role of Kir channels in K^+ homeostasis, impairment of this astrocytic function is expected in gliosis, and is in fact observable in the gliotic tissue surrounding an experimental epileptic focus (40). Accordingly, the loss of astrocyte Kir conductance, leading to high P_B/P_{BH^+} values for ammonia and diminished modulation of pH_i by $[K^+]_o$, will impair also the potassium-ammonium countercurrent mechanism proposed here. It would be valuable to assess the nature and extent of the decrements in CNS function attributable to impairment of astrocyte-mediated K^+ homeostasis both in hyperammonemic syndromes and in the aging brain. The isolation of glia-specific Kir channels (16) brings this goal closer to attainment.

6. CONCLUSIONS

Evidence of rapid permeation of NH_4^+ ions in astrocytes in vitro raises the possibility that the distribution of ammonia in the brain is determined not only by the pH of intracellular and extracellular compartments, but also by membrane potential-depen-

dent accumulation of NH_4^+ in astrocytes. Based on this premise, intracellular NH_4^+ concentrations in astrocytes *in situ* should not be uniform, but should vary as a function of local neuronal activity. This linkage derives from activity-related fluctuation of $[\text{K}^+]_o$, together with the role of $[\text{K}^+]_o$ as a regulator of (a) NH_4^+ permeation, (b) compartmental pH, and (c) NH_4^+ metabolism by glutamine synthase. In quiescent regions of faster NH_4^+ permeation, decreased pH_i , and decreased glutamine synthase activity, the local intracellular NH_4^+ concentration in astrocytes rises. In active zones, where $[\text{K}^+]_o$ is high, opposite trends decrease intracellular NH_4^+ concentration. Consequently, intracellular NH_4^+ in the astrocyte syncytium migrates towards zones of neuronal activity as intracellular K^+ migrates away. The molecular identification of the Kir channels that are a probable major pathway for NH_4^+ permeation in astrocytes will facilitate a more direct examination of the premises upon which this concept of a potassium-ammonium countercurrent is based, and permit an evaluation of impaired K^+ homeostasis associated with reactive astrogliosis in aging and disease.

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V

ASTROCYTES AND THE
BLOOD-BRAIN BARRIER IN AGING

Molecular Anatomy of the Blood-Brain Barrier in Development and Aging

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1. INTRODUCTION

The blood-brain barrier (BBB) is characterized by bidirectional exchange mechanisms which occur at the morphological interfaces separating the intravascular compartment from the brain parenchyma.

The cerebral endothelium plays a crucial role in maintaining the BBB properties. The morphological equivalents of the endothelial barrier are represented by two structural components: nonfenestrated endothelial cells, which are sealed by impermeable tight junctions (1,2) and a low appearance of endocytotic vesicles within the endothelium. In addition to the cerebral endothelium further structural components serve a function as a secondary barrier:

1. Pericytes, which cover the endothelial cells separated by a perivascular basement membrane from the endothelium.
2. The basement membrane itself, which surrounds both, the pericytes and the endothelial cells. The basement membrane plays a protective role and functions as an electrostatic selective filter for charged macromolecules (3).
3. Astroglial endfeet, which are actively involved in maintaining the BBB properties.

In addition to the structural components, various enzymes, transporters, and receptors within the cerebral microvasculatur provide the biochemical and metabolic constituents of the BBB, which regulate the transport of various biological substrates, such as ions, nucleotides, amino acids, glucose, peptides, and hormones (4).

2. DEVELOPMENT AND AGING IS ASSOCIATED WITH CHANGES IN THE BBB PHENOTYPE

In fetuses and newborns the BBB is immature, which means that it lacks structural and functional properties prevalent in the mature brain.

At embryonic day 11 (E11) of rat, the first blood vessels invade the brain anlagen from the perineural plexus and at embryonic day 12–14 (E12–14) the first intracerebral blood vessels become visible. At this time perivascular glia is undifferentiated. At embryonic day 15 the closure of the BBB tight junctions occurs and thereafter, intracerebral angiogenesis proceeds within the differentiating brain tissue concurrent with the increase of volume of brain parenchyma.

The formation of endothelial tubes is accompanied by association of locally-derived mesenchymal cells that are prone to either differentiate into pericytes (in capillaries and postcapillary venules) or smooth muscle cells (in larger vessels).

The ensheathment of cerebral microvessels by perivascular astrocytic endfeet is a late event in BBB development occurring postnatally around week 2–3 (5), followed by completion of the basement membrane (week 3–4 postnatally).

This brief description of the schedule of BBB differentiation shows that maturation of the BBB is a sequential process that follows a well-defined morphogenetic program.

Our review is aimed to summarize relevant data on the morphology and function of the BBB in the developing and aging brain.

3. AGE-RELATED STRUCTURAL CHANGES OF THE BLOOD-BRAIN BARRIER (BBB)

Age-related changes of structural components of the BBB include alterations of the microvascular and perivascular complexes of the BBB (for extensive reviews *see* 6,7,8,9).

Throughout aging and senescence the number of endothelial cells is steadily reduced, the endothelium is elongated (10,11), and the thickness of the subendothelial basement membrane is increased (12). The development of tight junctions can be monitored by the expression of tight junction associated protein (ZO-1). ZO-1 is a peripheral membrane protein and is concentrated at the cytoplasmic surface of tight junctions (13). It is a reliable indicator for the presence of tight junctions between cerebral endothelial cells (14). Occludin, an integral membrane protein, has been identified as a further component of tight junction strands (15). The expression of occludin is developmentally regulated. It is low in rat brain endothelium at postnatal day 8 but clearly detectable in adults (16). Recently, the claudin gene family was described to be directly involved in the barrier function of tight junctions (17) but no data on its expression in cerebral blood vessels have been published thus far.

An interesting aspect of BBB maturation is that the susceptibility of the BBB to pathologic agents differs with age. For instance, susceptibility to mumps-associated hydrocephalus is age-dependent and considered to be related to the maturity of the BBB. In hamster 2-d-of-age occurrence of pathologic signs are associated with the immaturity and fragility of endothelial tight junctions as evidenced by the determination of endothelial ZO-1 immunoreactivity (18).

In addition, besides changes in the classical features of the BBB complex (i.e., endothelial tightness and integrity of the metabolic complement) alterations of secondary structures like pericytic engulfment and the organization of perivascular astrocytes are essential for determining BBB integrity.

During aging the number of pericytes is increased and the cells show signs of degeneration with a high rate of inclusion bodies and vacuoles (19,20).

Concomitant, the number of astrocytes is increased (20) and the glial fibrillary astrocytic protein (GFAP) reveals enhanced expression (21) accompanied by a reduction of polarity of astrocytes (22). The functional meaning of these changes is essentially unknown, but studies on null mutations (i.e., GFAP $-/-$ mice, (23)) will help to clarify the role which the diverse structural components play in the concert of BBB preservation.

Table 1
Age-related structural changes of the blood-brain barrier

Component	Age related changes	Species-Region	Reference
<i>Microvascular part of BBB</i>			
Endothelial cell	Number is reduced, cells are elongated	Rat cortex	(10)
		Human cortex	(11)
Basement membrane	Increased thickness	Rat hippocampus	(12)
Pericyte	Degeneration	Rat	(19)
	Increased number	Rat	(20)
<i>Perivascular part of BBB</i>			
perivascular space	Formation of lacunae	Human	(25)
Astrocyte	Increased number	Rat	(20)
	Increased GFAP expression	Human	(21)
	Reduced polairty	Rat optic nerve	(22)
Microglia	Increased number of activated microglia	Human	(24)

The microglia shows no changes of its gross morphology. But there were significant age-associated increases in the total numbers of activated microglial cells, as evidenced by interleucin-1 alpha-immunoreactivity (24). A summary of the age-related structural changes of the BBB is given in Table 1. In the context of structural alterations it is important to emphasize that these changes of the BBB in aging are region- and species-specific.

4. AGE-RELATED FUNCTIONAL CHANGES OF THE BBB

4.1. Cerebrospinal Fluid and Serum

By comparing the protein-concentrations in the human cerebrospinal fluid (CSF) and blood serum, age-related changes in the function of the BBB can be assessed. Data from Garton et al. (25) and Kleine et al. (26) indicate that in human from 12th yr of age to adulthood the CSF/serum ratio for prealbumin, albumin, immunoglobulin G (IgG), and α 2-macroglobulin increases. It was taken into account, however, that the age-related changes of the CSF/serum quotients, with exception of α 2-macroglobulin, may also result from the decrease of blood serum protein concentrations. Therefore it was suggested that α 2-macroglobulin is the most sensitive indicator of age-related changes of BBB permeability (25). In children the CSF-concentration of albumin and IgG decreases from birth until 7-mo-of-age subsequently, thereafter a steady increase can be observed until 3-yr-of-age. Transthyretin and transferrin show an increased CSF/serum ratio until about 3-yr-of-age and decrease from then on (27). These differences in CSF protein concentration may reflect a phasic evolvement of BBB permeability including differential expression of receptor complements. A summary of age-related functional changes of the blood-brain-barrier indicated through CSF/serum analysis is given in Table 2.

Table 2
Functional changes in the BBB with aging. Permeability of the BBB as determined by CSF/serum ratio of individual proteins

Protein	Age related change	Reference
Prealbumin Albumin IgG α 2-Macroglobulin	Increased CSF/serum ratio correlate with age (adults aged 18–89 years)	(25)
Albumin α 2-Macroglobulin	Increased CSF/serum ratio correlate with age (child/adults aged 12–90 years)	(26)
Albumin IgG	Decreased CSF concentration from birth until 7 mo age	(27)
Albumin α 2-Macroglobulin Transthyretin	Increased (slightly) CSF concentration from 7 mo until 3 yr age	(27)
IgG	Increased (strongly) CSF concentration from 7 mo until 3 yr age	(27)
Transthyretin Transferrin	Increased CSF/serum ratio until about 3 yr and decreased from then on	(27)

4.2. Cerebral Endothelium

Substrate movement through the lipid bilayer of the cerebral endothelium is mediated by four mechanisms: Passive diffusion, facilitated diffusion, active transport, and receptor-mediated endocytosis accomplished by transport systems. In the following a brief account of the developmental- and age-related differentiation of these four mechanisms is given.

4.2.1. Transport- and Carrier-Systems

4.2.1.1. **GLUCOSE TRANSPORTER** Most BBB transport systems appear to be operational at birth (28). One specific example is the glucose transport system. The brain endothelial glucose transporter 1 (Glut 1), in its 55 kDa vascular isoform, is a well characterized intrinsic endothelial membrane glycoprotein which mediates the translocation of glucose across the plasma membranes of the cerebral endothelium. The expression of the glucose transport system is developmentally regulated and coincides with the maturation of tightness of cerebral microvessels (29,30,31).

4.2.1.1.1. *Expression of Glut 1 in the Immature State* During early angiogenesis a phasic expression of glucose transporter was observed in brain. While the neuroepithelium in rats at embryonic day 12 and 13 shows a high Glut 1 concentration, successive vascularization of the brain anlage and development of vascular tightness is accompanied by a significant reduction of Glut 1 expression in neuroepithelial cells and confinement of Glut 1 expression to the cerebral endothelium.

The level of Glut 1 remains unchanged during the first postnatal week but increases during the next two wk in postnatal development to attain adult levels at P21. Interest-

ingly, levels of Glut 1 mRNA remain unchanged (30). However, the length of the poly(A) tail of Glut 1 mRNA, a determinant of mRNA translatability, decreases with age (32) indicative for a translational regulation of Glut 1 expression and alteration of Glut 1 protein turnover with age. Transport kinetics in rabbits have been shown to exhibit a lower capacity (V_{\max}), but no significant changes in the half-saturation constant (K_m) of the Glut 1 in newborns as compared to the 28 day animals have been found (33). The age related increase in V_{\max} is a function of increased numbers of transporters on the endothelial plasma membranes. This period correlates well with the increase in brain activity and metabolism between birth and P21–P30. Furthermore, the temporal profile of Glut 1 expression during the first postnatal week follows the time course of the most active angiogenetic period in rat brain development which occurs between P3 and P5 (34).

These findings indicate that the abundance of Glut1 transporter correlates with the development of cerebral blood vessels.

4.2.1.1.2. Expression of Glut 1 in the Mature State and Senescence The amount of glucose transporter does not change with senescence, whereas the brain uptake of glucose decreases with age. This suggests that the reduced BBB transport of glucose is due to reduction in kinetics and/or affinity of the transport system to glucose rather than to age-related endothelial cell drop-outs (35).

4.2.1.1.3. TRANSPORTERS FOR OTHER SUBSTRATES Nine separate transport systems exist in cerebral capillaries for amino acid transport (36).

The cerebral endothelium is further furnished with a set of transporter systems for ions such as sodium, potassium and phosphate, for cytokines, transferrin, several neurotransmitters and opiate peptides, as well as hormones (37). The brain substitutes its iron through transcytosis of iron-loaded transferrin across the brain microvessels. The transferrin receptor is a well characterized receptor which is localized at the luminal and abluminal plasma membrane of the cerebral endothelium (38). Immunocytochemical localization of transferrin and its receptor in the developing chicken brain shows immunoreactive neurons at embryonic day 10. Thereafter the immunoreactivity of the neuronal transferrin receptor becomes weaker around day 11–16 whereas the BBB vessels show increased positivity (39). In the adult brain neurons are negative for transferrin and its receptor (40). This schedule is similar to the phasic expression of the Glut 1 transporter system and speaks in favor of a general morphogenetic program that governs transporter expression during BBB maturation.

P-glycoprotein (PGP) is a product of the multidrug resistance 1 (*mdr 1*) gene which is believed to act as an active efflux system for endothelial cells. It effectively blocks the penetration of lipid-soluble drugs into the brain (41). PGP is highly enriched in adult brain vessels. Mice exhibiting a null mutation of the *mdr 1* gene (*-/-*) show an increase of BBB permeability (42). In rat brain PGP shows a late onset of expression. In prenatal brains and in early postnatal stages of development PGP is undetectable. It is first detectable around P 7 and increases gradually to reach plateau levels (42). The lack of PGP in the embryonic brain is of considerable clinical importance, explicitly when the intake of drugs which bypass the maternal-embryonic placenta barrier is considered. Apparently, the embryo and developing

fetus is unable to eliminate cytotoxic drugs and to prevent them from entering the cerebral parenchyma.

4.2.2. Enzymes

One example of a blood-brain barrier specific enzyme is γ -glutamyl-transpeptidase (γ -GT) which serves a major function in the transport system for large neutral amino acids in cerebral endothelium. The presence of astroglial cells is required for the expression of γ -GT in cerebral endothelial cells. Around the time of BBB closure γ -GT appears in rat brain capillaries (43). The first postnatal period (day 2–12) is characterized by a significant decrease in γ -GT activity. During the second phase of postnatal period (day 12–21) a fast increase in γ -GT activity can be measured. Between postnatal day 21 and 60 the enzyme activity acquires the adult level (44).

Several peptidases control the exchange of neuropeptides at the blood-brain or the brain-blood interfaces. A similar time course as described for postnatal activity of γ -GT is observed for aminopeptidase A, whereas alkaline phosphatase, a nonspecific phosphomonoesterase, dipeptidyl peptidase and aminopeptidase N increase steadily during the first 2 wk of postnatal development (45).

Monoamine oxidase (MAO) impedes the traffic of monoamine precursors into the brain by enzymatic breakdown of biogenic amines, thus preventing uptake of monoaminergic peripheral neurotransmitters into the interstitial cerebral environment. MAO is associated with endothelial cells. MAO activity also increases postnatally and reaches a peak at 3-wk-of-age (46). In aged rats alkaline phosphatase, monoaminoxidase B, and tau-glutamyltranspeptidase increase. On the contrary, lactate dehydrogenase activity and monoamine oxidase A decrease with age. No noticeable changes can be seen in acetyl-cholinesterase activity during aging.

A general concept of the pattern of enzyme activity at the BBB interface with respect to age-related changes cannot be drawn, in particular if interspecies differences are taken into account.

4.2.3. Receptors

A number of biologically active proteins, including immunoproteins such as IgG, hormones like insulin and N-tyrosinated peptides are actively transcytosed through cerebral endothelium via receptor/carrier mechanisms (47).

Insulin for example has limited access to the adult brain but has increased availability to the newborn brain where it may function as a neonatal brain growth promoter (48). The binding site for insulin at the BBB is the insulin receptor on the capillary endothelium. Whether the binding capacity of the receptor or the receptor density is responsible for the lower amount of insulin in adult brain is still unknown.

Aging in mammals is characterized by a decline in plasma levels of insulin-like growth factor 1. The cerebral vasculature is an important source of insulin-like growth factor 1 for the brain. With age there are no changes in mRNA expression but the protein level decreases. There is also no change in type 1 insulin-like growth factor receptor mRNA with age but the receptor decrease in several brain regions (49). As already indicated for Glut 1 expression age-related adaptation of BBB protein levels seems to be primarily regulated through translational or posttranslational regulation.

4.2.4. Cell Adhesion Molecules

Besides the classical receptor/enzyme complement of the BBB some additional functional moieties like cell adhesion molecules exhibit age-related expression patterns. For instance, the platelet endothelial cell adhesion molecule-1 (PECAM-1) plays an important role in immune responses of the brain. In mouse BBB PECAM-1 is initially upregulated prior to structural maturity of the BBB with an increase up to day 7–10 postpartum and then decreases two weeks after birth. These findings suggest that the development of an “immune” BBB manifests prior to anatomical closure of the BBB (50).

The adhesion molecule ICAM-1 shows the same upregulation prior to the structural maturity of the BBB on the luminal and abluminal endothelial surface and a downregulation two weeks after birth (51).

An endothelial specific antigen with unknown function is the MECA-32 (mouse endothelial cell antigen). It is expressed during embryogenesis in the mouse brain microvasculature. This antigen is lost during the differentiation of the BBB, but remains present on the brain vasculature that does not exhibit barrier characteristics, i.e., choroid plexus and circumventricular organs. These findings suggest that MECA-32 is involved in maintaining the fenestrated phenotype in leaky brain capillaries (52).

4.2.5. Nonendothelial Components Associated with the BBB

The cerebral vascular bed contains additional structural components, which serve complementary functions in maintaining the metabolic BBB complex.

A predominant role among these additive components is played by perivascular cells (pericytes) which are distinctively shaped forming numerous cytoplasmic processes that encircle endothelial cells. Pericytes are separated from the cerebral endothelium by a common basement membrane. They take up blood-borne substances through phagocytosis (67) and they are identified as immunocompetent antigen-presenting cells (68), which participate in angiogenesis and are suggested to play a regulatory function in the control of capillary growth (69).

Cerebral pericytes constitute a significant secondary element in metabolic BBB functions (70). Immunocytochemical studies provide evidence for the existence of a BBB-specific enzymatic machinery in pericytes which exhibits γ -glutamyltranspeptidase, alkaline phosphatase, aminopeptidase A, and aminopeptidase N. Pericytes reveal a developmentally regulated pattern of their enzymatic complement similar to the differentiation of endothelial BBB constituents during development. The onset of γ GT-expression in embryonic rat brain occurs on E15 accompanied by closure of the BBB whereas the first noticeable expression of pericytic aminopeptidase N (pAPN) occurs on day E18. A steady-state level is reached at day 6–8 postnatally (71).

In summary, the described developmental changes in BBB phenotype are subject of morphogenetic control mechanisms that regulate the maturation of cerebral blood vessels. These mechanisms are prevalent from early embryonic angiogenesis to post-natal stages and exhibit a phasic schedule. Susceptibility of the developing BBB to pathogenic agents is regarded to be phase specific. Alterations of the aging BBB are manifold including increase in serum proteins as well as changes in the kinetics and affinity of transporters to their substrates. However, the molecular mechanisms which underly the functional deficits of the aging BBB are essentially unknown and remain to be elucidated by a cooperative structural/molecular approach.

Table 3
Summarizes the systems governing the blood-brain exchange and shows the effects of development and aging on the exchange rate

Transport System	Development and age related change	Species	Reference
<i>Transporters</i>			
Glucose Transporter 1	decreased (day 1–14)	Rabbit	(53)
	increased (day 14–70)	Rabbit	(33)
	increased (day 1–30)	Rat	(30)
Glucose Transporter 1 mRNA	unchanged	Rabbit	(53)
		Rat	(32)
Neutral Amino Acids	unchanged	Human	(54)
Tryptophan	decreased	Rat	(55)
Arginine	decreased	Rabbit	(56)
Adenine	decreased	Rabbit	(56)
P-Glycoprotein	increased	Rat	(42)
<i>Enzymes</i>			
Adenylate cyclase	decreased	Mouse	(57)
Aminopeptidase A	decreased (week 1–2)	Rat	(45)
	increased (week 2–8)		
Aminopeptidase M (N)	increased (week 1–8)	Rat	(45)
Alkaline Phosphatase	increased (week 1–2)	Rat	(45)
	increased	Rat	(58)
	increased	Mouse	(59)
	increased (week 1–8)	Rat	(45)
Dipeptidyl-Aminopeptidase IV	increased	Rat	(58)
Gamma-Glutamyl-Transpeptidase	increased (week 1–8) (fourfold)	Rat	(45)
	increased (day 10–180)	Rat	(59)
	decreased (day 180–360)		
	decreased (day 2–12)	Rat	(44)
	increased (day 12–21)		
	increased (day 7–month 12)	Mouse	(60)
	decreased (month 12–)		
Lactate Dehydrogenase	decreased	Rat	(58)
Monoaminoxidase	increased (day 1–14)	Rat	(46)
	decreased (day 14–)		
Monoaminoxidase A	decreased	Rat	(58)
Monoaminoxidase B	increased	Rat	(58)
Small Peptides	increased	Rat	(61)
Choline	decreased	Human	(62)
Cholinesterase	increased	Rat	(63)
Protein Kinase C β -Isoform	unchanged	Rodent, Rat, Human	(64)
	decreased	Human-Alzheimer Disease	(64)

(continues)

Table 3
(Continued)

Transport System	Development and age related change	Species	Reference
Transferrin	Shift from neurons to blood vessels	Chicken	(40)
<i>Receptors</i>			
β -Adrenergic	decreased (mo 3–24)	Rat	(57)
Insulin-like growth factor 1 receptor mRNA	Unchanged	Rat	(49)
Insulin-like growth factor 1 Protein concentration	decreased		
Insulin-like growth factor 1 receptor level	decreased (in hippocampus and cortical layers II/III and V/VI)		
<i>Antigens</i>			
Neurothelin (HT7)	Shift from neuroblasts to endothelial cells	Chicken	(65)
Intercellular-adhesion-molecule (ICAM-1) (CD54)	increased (day 2–7), decreased (day 7–14), no expression (day 14–)	Mouse	(51)
Platelet-endothelial-cell-adhesion-molecule (PECAM-1) (CD31)	See ICAM-1	Mouse	(50)
(neural)-N-Cadherin	decreased (with the onset of BBB differentiation)	Chicken	(66)
Occludin	Low level (day 8), increase (day 70)	Rat	(16)
MECA-32	Until E16, decreased (E17–)	Mouse	(52)

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The Blood-Brain Barrier in the Aging Brain

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1. INTRODUCTION

Homeostasis of the extracellular microenvironment in the neural tissue of the brain as well as its protection against neurotoxic compounds and variations in the composition of the blood are important for normal function of the neurons. It is warranted by a structure formed between blood and brain which is therefore called the blood-brain barrier (BBB). This barrier has been postulated in earlier decades by experiments using dyes which directly visualized both the protection of the brain if injected into the vasculature, and free access of the brain if injected into the cerebrospinal fluid. Since in the first experiment some areas of the brain around the ventricle were stained and in the second experiment the identical areas were not, the barrier necessarily had to be separated into a BBB and a blood-cerebrospinal fluid barrier.

In most vertebrates, the BBB is located in the brain capillary endothelial cells (1; for a review, *see* ref. 2). Where the endothelial BBB is leaky in order to gain access for neurosecretory neurons to the vasculature, a barrier has to be formed to avoid free substance exchange between blood and cerebrospinal fluid. These barrier cells in the so-called circumventricular organs are glial tanycytes, which are in line with the periventricular ependymal cells (3). As well, the plexus epithelial cells of the choroid plexus are destined to produce the cerebrospinal fluid from the blood. The blood vessels in these areas are fenestrated (4), and the fenestration is now known to be induced by the vascular permeability factor (VPF) which is identical with the vascular endothelial growth factor (VEGF) secreted by, for example, the choroid plexus epithelial cells (5). At the surface of the brain, there is a third type of barrier, the outer blood-cerebrospinal fluid barrier which protects the brain against blood-borne influences from leaky vessels of the dura mater. This outer blood-cerebrospinal fluid barrier is located in the meningeal cells (6, 7).

The endothelial, glial, and meningeal subtypes of the plasma-interstitial fluid barrier, which together form the BBB in a wider sense, have a common feature: A dense meshwork of intramembranous strands occluding the intercellular cleft. These tight junctions (TJs), together with a low number of endocytotic vesicles, are generally believed to represent the basis of low transcellular permeability (1,8). If considering the BBB properties of the aging brain we have to look both at those structures which are responsible for the low permeability and at those cells which are suggested to

induce it. In a first part, we will briefly describe BBB properties of the normal brain vasculature including endothelial TJs, receptors, transporters, and enzymes as well as the capillary-associated cells, and in a second part, we will summarize alterations of the BBB during aging.

2. PROPERTIES OF THE BLOOD-BRAIN BARRIER

2.1 The Endothelial Barrier

Mature BBB capillaries in the mammalian brain are mainly characterized by the small height of endothelial cells, the complex interendothelial TJs, the small number of caveolae at the luminal surface of the cell (4), and the high number of endothelial mitochondria (9). In addition, subendothelial pericytes which are completely surrounded by a basal lamina, phagocytic perivascular cells and astrocytic processes belong to the set of elements directly adjacent to the cerebral vasculature (10,11)

The microvascular endothelial cells are doubtless most important in the determination of the BBB-related permeability. From transplantation experiments it became evident that BBB characteristics are determined to a great extent by intrinsic factors of the brain (as summarized in ref. 12). Initially, proliferating and migrating endothelial cells are suggested to be committed to express the BBB phenotype by the interaction with neuroectodermal cells (13). The expression of N-cadherin on endothelial cells invading the neuroectoderm has recently been proposed to represent an initial and transient signal involved in this commitment (14). Consecutively, astrocytes would stabilize and maintain the acquired barrier properties. This proposal of BBB development is supported by *in vitro*-experiments showing that factors released from astrocytes seem to be necessary to induce BBB characteristics (15). However, they do not seem to do so in a quantitatively sufficient manner (16,17). Nature and mechanism of the putative BBB-inducing factors remain elusive.

The morphology of TJs as the elements restricting the paracellular permeability still is most suitably investigated by means of the freeze-fracture technique. In epithelia, the TJ strands are almost completely associated with the protoplasmatic fracture face (P-face). On the external fracture face (E-face), the grooves, which are mainly free of particles, may represent imprints caused by the P-face-associated strands. It is conspicuous that in rat brain endothelium *in situ*, the P-face association of TJ particles is significantly lower than in epithelia and higher than in non-BBB endothelia (15). Since in cultured BBB endothelial cells both the electrical resistance and the P-face association of the tight junction particles are reduced, it was speculated that the extent of the P-face association may inversely correlate with the transendothelial permeability (17,18).

Regarding the molecular composition of TJs, there are two competing hypotheses: the lipid model proposes inverse lipidic micelles to represent the TJ strands (19,20) and implies the continuity of the external leaflets of two adjacent cells. The protein model suggests the insertion of proteins into the membrane occluding the intercellular cleft (21). Indeed, four-transmembrane domain protein components of the TJs, occludin and the claudins, were identified and characterized so far (18,22–25). Transfected cells expressing claudin-1 form TJs with a high P-face association resembling those in epithelial cells, whereas transfectants expressing claudin-2 develop TJs with E-face-

associated chains of particles resembling TJs in peripheral endothelial cells or BBB endothelial cells in vitro (23). In the central nervous system (CNS), the choroid plexus was the only site where claudin-2 was detected (26). The claudins found to be expressed in the CNS thus were claudin-1, claudin-2, claudin-5 and claudin-11. Claudin-11 was identified as the oligodendroglia-specific protein OSP (27); consequently, the claudins 1 and 5 are specific for brain endothelial cells. Indeed, Morita et al. (28) were able to characterize claudin-5 as endothelial-specific. Furthermore, the authors transfected fibroblasts with the cDNA of claudin-5 and found that the particles of the TJs that were formed in these transfected cells were associated with the E-face. Since the BBB endothelial cells show variable degrees of the association with the P- or E-face under different conditions, we are tempted to speculate that the permeability of the BBB TJs may be regulated via the stoichiometry of endothelial claudins 1 and 5. This view is supported by the observation that in human astrocytomas the BBB is dysregulated, the endothelial TJs are predominantly associated with the E-face, and the expression of claudin-1 is downregulated (29).

In the last years, several TJ-associated proteins were identified such as ZO-1, ZO-2, ZO-3, 7H6 and cingulin. All these proteins are under the control of different kinases, and their phosphorylation state correlates with barrier function (as summarized in ref. 15). In addition, there exists an interplay between tight and adhesion junction components which is not well understood so far.

The TJ-based paracellular impermeability of the brain capillary endothelial cells implicates that hydrophilic substances that are essentially needed for the metabolism of the brain require transport through the endothelial wall. Several independent carrier systems for the transport of hexoses (glucose, galactose), neutral, basic and acidic amino acids, monocarboxylic acids (lactate, pyruvate, ketone bodies), purines (adenine, guanine), nucleosides (adenosine, guanosine, uridine), amines (choline), and ions have been described (for details, *see* 12,30). Among these transporters, the glucose transporter is of special importance due to the fact that glucose is the main energy source of the brain. The 55kDa form of the glucose transporter isoform Glut1 as one of five members of a supergene family of sodium-independent glucose transporters is highly restricted to the capillary endothelial cells in the brain (31). This restriction to endothelial cells (32) and the asymmetrical distribution in the endothelial cell membranes (the density of transporter molecules is four times higher in the abluminal than in the luminal membrane) are believed to reflect the onset of BBB function during development (33,34).

In addition to the carrier-mediated transport from blood to brain, the receptor-mediated transport has also been investigated intensively. In BBB endothelial cells, receptors for the endocytosis of transferrin, LDL, immunoglobulin G, insulin and insulin-like growth factor have been described (12,35–38). Furthermore, a large number of receptors which are by no means specific for brain endothelial cells (for example for adrenalin [α and β], histamin, endothelin, adenosine as well as for angiogenic growth factors such as fibroblast growth factor, vascular endothelial growth factor or angiopoietin) has been described to participate partly in the regulation of BBB-specific enzymes (30) or to be responsible for the induction of angiogenesis (39–41). Enzymes associated with the BBB such as the adenylate cyclase, the alkaline phosphatase, the nonspecific phosphomonoesterase, the Na^+/K^+ -ATPase, the NO-synthase, the guany-

late cyclase or the cholinesterase have been suggested to play an important role in the regulation of transendothelial permeability or glio-vascular interrelationships (30,42), whereas the DOPA-decarboxylase and the monoaminooxidase are connected to the so-called metabolic BBB which restricts the transfer of L-DOPA between brain and blood by enzymatic conversion within the endothelial cell (43). Other enzymes such as the γ -glutamyl-transpeptidase and antioxidant enzymes (glutathione peroxidase, glutathione reductase, catalase, and superoxid dismutase) are involved in the transfer of γ -glutamyl residues of glutathione to amino acids or the cleavage of the reduced form of glutathione (44) and the protection of the BBB against free radical peroxidation (45). Some of these enzyme activities decline during aging (*see Subheading 3.1.*).

There are still other antigens associated with the BBB. For example, in order to restrict free diffusion also of lipophilic compounds from blood to brain, a control mechanism is required to export them effectively out of the brain endothelium. In this context, the P-glycoprotein which is a transmembrane protein that confers multidrug resistance is of special importance. P-glycoprotein (46) as well as the multidrug resistance-associated protein Mrp1 (47) have been identified in the brain endothelial cell membranes. Moreover, an important class of surface molecules mediating the interaction between blood cells and endothelium represent the adhesion molecules. Whereas the function of neurothelin/HT7, a member of the immunoglobulin superfamily (48,49), is still unclear, the expression and upregulation of the intercellular and vascular cell adhesion molecules (ICAM-1 and VCAM-1, respectively) have been identified as essential steps for lymphocyte recruitment during inflammation (50,51).

2.2. Interactions Between Endothelial Cells and Glial Cells or Pericytes

The extracellular space between the vascular complex and the astrocytes is extremely narrow (52), and the membrane of astrocytes directly adjacent to the blood vessel is crowded with a high density of so-called orthogonal arrays of particles (53). Recently, it was found that the water channel-protein aquaporin-4 is a principal constituent of these arrays (54,55). A typical feature of astrocytes *in situ* is the high polarity of astrocytes: It is characterized by the polar distribution of both OAP and aquaporin-4 and has been proposed to be an essential constituent of the BBB *in vivo* (56).

The endothelial basal lamina is believed to play a crucial role for the maintenance of the BBB. The composition of the basal lamina includes many compounds of the extracellular matrix (ECM) such as fibronectin, laminin, type IV collagen and glycosaminoglycans. Since the ECM of cerebral microvascular vessels changes dramatically during aging, it is important to test the influence of single ECM-components on the BBB directly. Nevertheless, detailed information about that are still lacking so far. Agrin which is a heparan sulfate proteoglycan originally discovered at the neuromuscular junction and more recently shown to be committed to multiple functions in the CNS (survey in ref. 57,) was found to be accumulated in the brain microvascular basal lamina. Because of the parallel onset of agrin expression and BBB development, and the strict localization with BBB endothelial cells, it was suggested to be important for its formation and maintenance of the barrier (58).

The role of other vessel-associated cells such as pericytes, perivascular cells or microglial cells in the formation and maintenance of the BBB is unknown or still circumstantial. The pericytes seem to control the microvasculature by expressing recep-

Table 1
Age-related changes in permeability

	Human	Monkey, Rat, Mouse
Changes in permeability in aging	(111,112) ↔	(99,108,114)↔ (108) (in gray matter); ↑ (in white matter)
Changes in permeability in aging- associated diseases	(112,113) Alzheimer's disease ↑ Vascular dementia ↑↑	Hypertension: (99,115,116)↑ Ischemia: (66,117)↑

↔ no change; ↑, increase; ↓, decrease;

tors for catecholamines, endothelin-1, vasoactive intestinal peptide, vasopressin and angiotensin, and they may play an important role in the process of inflammation (11). BBB pericytes have been regarded as a second line of defense (59) which is reflected under pathological conditions by the increase of pinocytosis and of enzyme activities which normally are characteristic of endothelial cells (11). The pericyte:endothelial cell ratio is four times higher in the retina than in the brain (60), but generally higher in the CNS when compared with other organs (11). In platelet-derived growth factor (PDGF)-B-deficient mice, Lindahl et al. (61) reported the loss of pericytes and the formation of microaneurysms. This can be explained by PDGF-receptors expressed by pericytes. If the close interplay between pericytes and endothelial cells which is—for example—represented by the expression of angiopoietin-1 by pericytes and its receptor tie-2 by endothelial cells, is disturbed by tie-2- or angiopoietin-1-null mutation, the mice fail to develop a functional integrity of blood vessels (62,63).

3. ALTERATIONS OF THE BLOOD-BRAIN BARRIER DURING AGING

After the description of the morphological, molecular, and functional properties of the BBB of the adult brain, we now will have a look at the alterations taking place during aging. First of all, we have to state that the permeability of the BBB in the intact aged brain seems not to be increased. However, several authors claimed that the susceptibility or vulnerability of the BBB to factors compromising the cellular integrity of the vascular wall is increased during aging. For example, Sankar et al. (64) could show that amphetamine induces an increase in permeability to albumin in aging rats compared to younger control animals suggesting an age-related deficiency to compensate environmental stimuli. Under pathological conditions, such as hypertension, ischemia, Alzheimer's disease, an increase of the BBB permeability has been described which could lead to severe brain edema (for literature, see Table 1). The BBB breakdown may be due to a higher rate of transcytotic vesicular transport of high molecular weight proteins (65) which could be induced by the release of serotonin from blood platelets ((66); compare also the serotonin-dependent extravasation of ferritin across venules via formation of vesiculo-vacuolar organelles, as described by Feng et al. (67)). A destruc-

Table 2
Age-related changes in CNS microvasculature

Components	Human	Monkey	Rat
Cross-sectional area of capillary wall	(118) ↑	(76) ↓	<i>Hippocampus:</i> (100) ↓ <i>Cortex:</i> (99,100) ↓ (120) ↓ (76) ↔
Number of endothelial cells	(118) ↓	(94) ↓	(120) ↓
Number of mitochondria	(68) ↔	(76,94) ↓	(120) ↔ (121) ↑
Gliofibrillary proliferation	(119) ↑	NR	NR
Thickness of basement membrane	(119) ↑	(76) ↑	(74,92) (76,94,100,103, 104) ↑
Changes in number of pericytes	(68) ↓	(98) ↔	(74,104) ↑ (103) ↓

↔ no changes; ↑, increase; ↓, decrease; NR, not reported

tion of TJs could neither be observed nor ruled out. Thus, an increased BBB permeability is not a necessary consequence of aging, but seems to reflect a restricted ability to react on unfavorable conditions such as inflammation, hypertension or high concentration of any neurotoxic compounds in the blood. There are many details known about alterations of structures or enzymatic activities in the different cells of the BBB in the aged brain (30,68–74) but essentially less is known about which change is causally connected to which functional inferiority. Investigations have been carried out not only in normally aged animals or human beings, but frequently in the senescence-accelerated mouse strain (SAM) which was inaugurated as a model system by Takeda et al. (75; for a recent review, see ref. 73).

3.1. Age-Related Alterations of the Endothelial Cells

There exists an enormous body of literature on age-related morphological changes of the CNS microvasculature including perivascular cells. Briefly, most authors described changes in the cross-sectional area of the capillary wall, the intercapillary distance, the number of endothelial cells, the number and density of mitochondria per cell, and the thickness of the subendothelial basal lamina (68,71,76–78). see Table 2 and Fig. 1. That the information about these parameters frequently are controversial, may be due to differences among the species (mostly human, monkey, and rat), the neuroanatomical site looked at, in whether venules, arterioles or capillaries were considered, and finally in the methodology. The mostly described morphological age-related change in the CNS vasculature concerns the thickening of the subendothelial basal

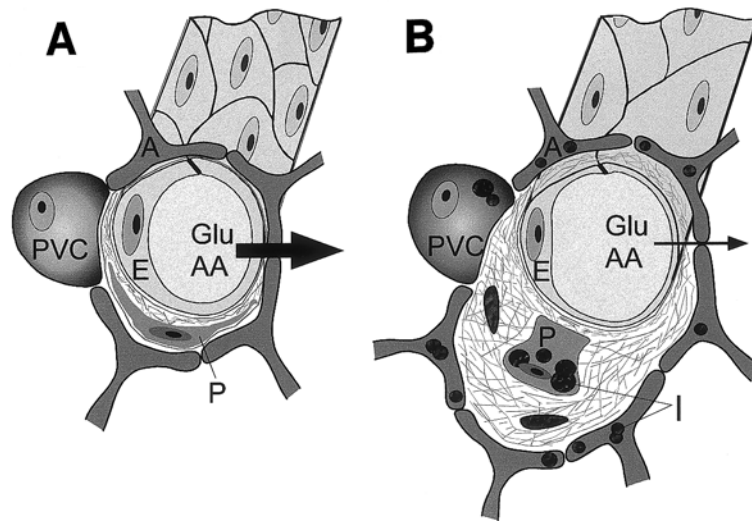


Fig. 1. Schematic view of a normal (A) and a brain capillary showing age-related changes (B). The most conspicuous alterations are the attenuation and elongation of the endothelial cells (E), the thickening of the basal lamina, the vacuolization of pericytes (P), perivascular cells (PVC) and astrocytic endfeet (A) and the appearance of inclusion bodies (I), and the decline of the transendothelial glucose (Glu) and amino acid (AA) transport (thin vs thick arrows; *see ref. (30) 1997*). Changes of the content of endothelial mitochondria was not referred to because of controversial data in the literature (*see Table 2*).

lamina (Table 2, Fig. 1). This increment might have consequences for the barrier regulation of endothelial cells. For example, Tilling et al. (79) reported on a decrease of the transendothelial permeability in cultured endothelial cells when grown on laminin, type IV collagen or fibronectin, when compared to rat tail collagen. The actual composition of the ECM, in particular the basal lamina, is dependent on matrix metalloproteinases, serine proteinases and their inhibitors which are normally present in the brain and differentially activated. MunBryce and Rosenberg (80) were able to show that after an intracerebral injection of type IV collagenase the vascular basal lamina was disrupted and the BBB opened. A growing body of literature presently describes the involvement of matrix metalloproteinases in the disruption of the BBB (81) suggesting that the degradation of the basal lamina and a breakdown of the BBB are causally related processes. If so, integrins could play a major role in the signaling pathway from the ECM components to the mechanisms which are involved in triggering the BBB permeability (82).

The effect of basal lamina thickening on endothelial permeability has been studied in organs other than the brain, but it cannot be excluded that in BBB endothelial cells, too, these results may be valid. For example, the nonenzymatic glycation of collagen probably is in part responsible for the age-related thickening of basal lamina and far-reaching pathological effects on the microvasculature. The first step of this reaction is the nonenzymatic condensation of glucose and the epsilon amino group of lysine,

which is followed by the formation of a Schiff base. This may be stabilized by an Amadori rearrangement from which in turn the nonenzymatic glycation is initiated (for a review, *see ref. (83).*) The resulting so-called advanced glycation endproducts (AGEs) are able to crosslink proteins. Because of the long half-life of many ECM proteins, AGEs can accumulate and alter the chemical and mechanical properties of these molecules. The glycation pathways are influenced by factors such as phosphate and glucose concentration, pH and oxidative stress. Well investigated AGEs are carboxymethyllysine (CLM), pentosidine, and pyrraline. Protein glycation and crosslinking of proteins increase in aging and diabetes (83–85). The crosslinked collagen might be responsible for the resulting insensitivity to proteases and the following thickening of the basal lamina. Interestingly, the accumulation of glycation products on matrix-components affects the behavior, phenotype, and intracellular signaling of resident cells. Haitoglou et al. (86) have shown that endothelial cells, grown on nonenzymatically glycated laminin and type IV collagen, reduced their spreading and adhesion. Accordingly, Hasegawa et al. (87) observed in endothelial cells, plated on glycated basement membrane-like matrix, both a reduced intracellular tyrosine phosphorylation of paxillin, a member of focal adhesion proteins, and a distribution of paxillin and actin filaments which were different to control cells. We now know that the matrix glycation affects the cells via receptors which bind AGEs and were identified by Vlassara et al. (88). This AGE-receptor is distinct from the receptor for AGE that was detected by the Stern group and called RAGE (89,90). Ligand binding by RAGE resulted in the induction of oxidant stress in endothelial cells and in increased permeability (91). The activation of these receptors can also result in the synthesis and release of cytokines such as IL-1 and TNA- α (88). However, it should be held in mind that most of these results were gained by investigating other than BBB endothelial cells. Nevertheless, Mooradian and Meredith (92) reported that the content of AGEs, namely pentosidine, in cerebral microvessels did not change with age. Therefore, the correlation of AGE production with thickening of the basal lamina and the increased permeability of the BBB should carefully be examined. Handa et al. (84) showed that the AGE pentosidine is effective in inducing the platelet-derived growth factor PDGF in retinal pigment epithelial cells *in vitro*. The authors speculate that AGE-induced changes in Bruch's membrane could alter the outer blood retinal barrier function. The β -amyloid protein which is deposited during normal aging around brain microvessels and increasingly in Alzheimer's disease has been found to bind to RAGE and to be involved in transendothelial migration of monocytes (93). Thus, the roles of glycation products and RAGE for the maintenance of the integrity of brain microvessels seem to be more relevant than thought previously, and the investigation of their pathological consequences in the context of BBB aging will be a challenge for the future.

The attenuation of endothelial cells is another parameter frequently described in the literature on aging. In an attenuated endothelial cell, the mitochondrial distribution and density obviously change, thus rendering it difficult to determine the true shift in the number of mitochondria per cell. Nevertheless, it seems generally accepted that mitochondrial content in brain capillary endothelial cells declines during aging (71,76,94). Possibly, this decline of mitochondrial content is causally related to an increased risk of the cells to be impaired by free radicals and lipid peroxides. Mooradian and Uko-

eninn (95) reported on an increase of lipid peroxidation by-products, such as malondialdehyde, in cerebral microvessels as a consequence of an age-related decrease of the antioxidative capacity. On the other side, Tayarani et al. (96) investigated antioxidative enzymes in aging brain capillaries and did not find a consistent decline of glutathione peroxidase and reductase activities. The activity of the superoxide dismutase was even increased with age; only the activity of catalase was reduced.

3.2. Age-Related Alterations of the Pericytes

During aging, pericytes show ultrastructural changes such as vesicular and lipofuscin-like inclusions (74,97–99); (Fig. 1), an increased size of mitochondria (100) and a foamy transformation (101). Ueno et al. (102) reported on membranous inclusions within the basal lamina or the ECM of the microvessels and suggested the degeneration of pericytes (Fig. 1). De Jong et al. (103) in the aging rat and Stewart et al. (68) in the aging human brain showed the degeneration and loss of pericytes. Burns et al. (76,94) described the reduction of the cross-sectional area of pericytes in aging monkey and rat brain. However, Peters et al. (98) found no change in the number of pericytes in monkey, and Heinsen and Heinsen (104) and Peinado et al. (74) showed an increase in the number of pericytes in the rat aging brain. The obvious discrepancies in the literature could be explained by considering the degeneration and the proliferation of pericytes as distinct age-related mechanisms. Accordingly, Scheibel and Fried (105) could show that pericytes in aging brain begin to lose their contact to the endothelial cell and begin to migrate. This behavior seems to be a response of the pericytes to a yet unidentified age-related effect. The PDGF released by endothelial cells is known to bind to the PDGF receptor expressed by pericytes and to attract pericytes to endothelial cells (106,107). If this ligand-receptor system is disturbed, the functional integrity of the vessel is impaired (61,107). With age, this system could deteriorate as well, but data are not available as yet.

4. PERSPECTIVES

The huge number of information on the normal and developing BBB comprising morphological, physiological, biochemical, immunological, pharmacological, and clinical data are far from being integrated into a commonly accepted scientific concept. Considering the data on the BBB in the aging brain, they are predominantly descriptive and less functional. They mainly concern morphological and enzymatic alterations and are considerably controversial. It would be very attractive to compare age-related changes with those observed in diabetes, gliomas or neurodegenerative disorders; however, due to the space available, this comparison has to be limited. In the first part, we gave a survey of the cellular and molecular components of the BBB and described, in the second part, some elected alterations in the aging brain. Finally, we wish to briefly delineate some strains of research which seem to us to merit interest in the future. Again, we are not able to cover all age-related changes of endothelial physiology and pharmacology. Namely, we did not refer to changes of transporters and receptors in aging, which have been reviewed by Shah and Mooradian (30) previously (but see Fig. 1).

First of all, although the knowledge of the molecular constitution of TJs as well as of interactions and interdependencies of junctional components connected to the bar-

rier formation in endothelial cells has considerably advanced, the characterization of these components in the aging brain still is difficult and equivocal. The permeability appears not to be substantially altered in aging brain, at least if considering high molecular weight compounds; regarding the permeability related to low molecular weight compounds, it seems to be slightly increased, at least in the white matter (108). The morphological parameters of endothelial TJs could be expected to change with age in a similar way as observed in glioma vessels or under pathological conditions (unpublished observations): This alteration of the TJ freeze-fracture morphology concerns the switch of the particles from the protoplasmic to the E-face, and this could mean an altered linkage to the cytoskeleton or a switch from claudin 1 to claudin 5 as has been already discussed by Tsukita and Furuse (18) and by Liebner et al. (29). Furthermore, the altered ECM (the thickening of the basal lamina, for example) and/or the nonenzymatic glycation of collagen are candidate factors influencing the permeability of endothelial cells (79,83,92). In particular, agrin was found to be widely expressed in senile plaques and neurofibrillary tangles in Alzheimer's disease (109). Interestingly, Donahue et al. (57) reported that in some cases of Alzheimer's disease the immunostain of agrin around vessels had a ragged profile and that senile plaques were seen frequently in the vicinity of capillaries. A large fraction of the agrin in Alzheimer's diseased brains changed biochemically into becoming insoluble in 1% SDS (57). The mechanisms by which agrin is involved in the maintenance of the BBB and by which the biochemical modification of agrin is involved in the breakdown of the BBB under pathological conditions such as Alzheimer's disease or tumor remain to be elucidated.

Although the activities of a number of endothelial enzymes are increased or decreased with age (30), no clear-cut concept on functional implications of these alterations has been proposed.

Similarly, the implications of the alterations of the pericytes are far from being elucidated. The understanding of the complex interplay between endothelial cells and pericytes is actually still at its beginning (11,14,61). Also, the role of astrocytes and the significance of the specific distribution of their orthogonal arrays of particles (corresponding to water channels which consists of aquaporin 4 (54,55,110) in the membranes of perivascular astrocytic endfeet is not elucidated. This distribution is a typical property of the mature BBB and lost or reduced in culture, after injury, in a glioma (53,56) and possibly during aging.

Since insight into regulatory mechanisms of the BBB are of primary significance for the development of new therapeutic strategies in neurology and neurosurgery, for instance for the treatment of gliomas or neurodegenerative disorders, the molecular analysis of these mechanisms in the developing adult and aging brain is highly important. The transfer of results from basic research on angiogenesis, adhesion molecules, junctional components, and the ECM to the field of research on aging will improve understanding age-related processes in the brain.

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Astrocytes and Barrier-provided Microvasculature in the Developing Brain

Luisa Roncali

1. INTRODUCTION

The morphofunctional specificity of the cerebral endothelia has been ascribed to the close association between these cells and the astrocytes, glial cells that in the central nervous system (CNS) microvasculature are arranged in an almost continuous perivascular sheath. The astrocytes are thought to play a role in inducing the tightness and other properties of the endothelial cells (the blood-brain barrier [BBB] that preserve the nervous tissue separate from the blood environment and guarantee the unique and privileged metabolism of the neuronal cells.

Many studies have been performed to investigate glial control on the differentiated status of the cerebral endothelia, starting from the suggestion by Davson and Oldendorf (1) that endothelial permeability via pinocytosis is restricted in the brain vessels by the perivascular glial action. In the cerebral endothelial cells, the low permeability, tightness of junctions, expression of specific enzymatic activities and transport systems, glucose uptake, as well as the cell membrane resistance and polarity, have been demonstrated to be influenced by astrocytes in various, mainly *in vitro*, BBB models (2–16). Although an intimate astrocyte-endothelium relationship has been shown to characterize the barrier-provided CNS microvasculature, whereas this association is absent where the brain vessels are barrier-free, such as in the circumventricular organs and in pathological situations of the brain, the inducing role exerted on cerebral endothelium by astrocytes is still under discussion (17–22).

Investigations carried out *in situ* on the developmental organization of the astrocyte-microvascular interface show that relationships between glial cells and blood vessels take place precociously in the CNS and that the first astroglial forms to send processes to the growing cerebral vessels are the radial glial cells. The radial glial cells, once referred to as matrix cells or spongioblasts, are transient cells committed to giving rise to multipolar astrocytes in the mature brain. Their body is located in the deep, ventricular layer of the nervous wall and they reach the external limiting membrane through a slender distal process that elongates and branches as the intermediate, mantle layer thickens. The radial glial cells are involved in several fundamental functions during CNS morphohistogenesis; among these they may provide a scaffolding in the nervous

wall for both neuroblast migration and the spatial arrangement of the first CNS vessels that grow radially from the perineural plexuses (23–26). Observations with both light and electron microscopy of cells of the glial lineage sending processes to the developing CNS vasculature date back to many years ago. Glial end feet attached to blood vessels are described at the 12th embryonic day in the chicken, at the 15th day in the mouse and rat fetuses, at the 48th embryonic day in the monkey and beginning from the 7th gestational week in the human brain (27–32). As soon as differentiation occurs, firstly of astroblasts and later of astrocytes, their processes also contribute to the building of the vessel glial sheaths that progressively widen as the perivascular space disappears. In the perinatal and adult brain microvasculature, the astrocytic coverage is almost complete (85% in the rat cerebral cortex; 96% in the optic *tectum* of hatching chickens; 86% in the human cerebral cortex), while other glial cells and neuron processes participate, at least to a small extent, in the composition of the perivascular sheath (33–36).

2. ASTROGLIA AND MICROVASCULATURE IN EARLY NEURAL DEVELOPMENT

The close, anatomical relationships established between astroglia and microvessels during early neural development suggest that astrocytes, as well as their precursors, may play a role in forming the blood-brain-barrier. Nevertheless, the perivascular glia lack barrier properties and constitute a blood-brain-barrier only in some invertebrates. During evolution, the endothelial barrier replaces the glial barrier, although the glial cells seem to maintain some barrier competence even in the vertebrate brain (37).

Developmental studies on the endothelial barrier have to be considered the most likely to provide information on the role of the glial cells in differentiation of the cerebral endothelium. In this context, it should be noted that whereas some investigations seem to indicate that the brain vessels are provided with barrier properties from the beginning of the neural angiogenesis, others suggest that the morphofunctional tightening of the endothelia takes place gradually during embryonic development (38,39).

Detailed results of this process have been obtained in the brain microvasculature of the chick embryo. The endothelial cells undergo continuous molding processes, involving their thickness and surface features, pinocytotic structures, junctional systems, and biochemical and antigen apparatuses. Only by birth does the chick endothelia seem to have achieved almost complete differentiation. By this time they have become flattened and smooth, with only few endocytotic vesicles and vacuoles, and are sealed by extensive pentalaminar junctions that appear as networks of interconnected fibrils on the replicas of freeze-fractured vessels (27,40–42). The vessel permeability to the classical markers, horseradish peroxidase and Evans blue, parallels the morphological changes in the endothelial cells since progressive restriction takes place that ends with complete blockage of vascular escape of the tracer by hatching time, for horseradish peroxidase, and by one month after hatching for the Evans blue marker (43,44). The time course of the metabolic maturation of the endothelial barrier investigated on isolated microvessels of the chick embryo brain is the same as that required for the anatomical differentiation of the endothelial cells. In fact in the endothelial cells there is a progressive decline in the activity of the alkaline phosphatase enzyme and in the expression of the transport systems for neutral aminoacids and D-glucose during embryonic life. They are further

reduced in the adult brain, in accordance with the increased tightness of the endothelial junctions (45). Among the cerebral endothelium antigens, the HT7 glycoprotein was recognized to be specifically expressed in the chick brain endothelial cells, where it correlates with the glucose transporter activity and BBB functioning (46). Like other barrier markers, HT7 expression modifies during chick embryo brain development, becoming specifically localized on the endothelial luminal and lateral surfaces at the late embryonic stages and in adulthood. The glycoconjugates of the endothelial cell plasma membranes also undergo changes during maturation of the BBB in developing chick brain vessels. N-acetylglucosamine and sialic residues, specifically located on the luminal surface of the endothelial cells and expressing their polarity, increase significantly in number parallel with morphofunctional differentiation of the endothelial cells (47,48).

The main morphological changes undergone by the endothelial cells of the mouse embryo and fetus brains are similar to those demonstrated in the chick embryo brain endothelia, and develop according to the same time sequence, mouse gestation being quite as long as chick incubation. In addition, in the mouse fetal brain, ZO-1 phosphoprotein expression, which correlates with the maturity of the tight junctions, modifies during development and shows a selective localization in the endothelial cells only by the prenatal age (49).

Recent studies carried out on the morphological characteristics of the microvasculature of the human fetus telencephalon further support the concept that the endothelial cells evolve toward a BBB status in the prenatal age (Fig. 1a, b) (50,51). This possibility is substantiated by some aspects of endothelial cell function, such as glucose transport and permeability to endogenous albumin. The glucose uptake by the barrier-provided microvasculature is mediated by the glucose transporter isoform 1 (GLUT1) whose expression in the endothelial cells is developmentally regulated (52,53). In the human telencephalon microvessels, GLUT1 antigen sites already show the specific localization on the abluminal and lateral endothelial plasma membrane by the 12th gestational week (54–56). The prenatal GLUT1 expression correlates with the efficacy of the endothelial barrier to the endogenous albumin (Fig. 2a, b) and with the expression of the multidrug-resistance P-glycoprotein (57,58).

Besides the endothelial cell characteristics, the cerebral microvessel specificity and BBB functioning is also because of the subendothelial pericytes and basal lamina. The CNS pericytes are contractile and phagocytic cells that express specific surface and cytoplasmic markers as well as enzymatic activities, and play vasodynamic, hemostatic and immunological roles (59). Pericytes escort cerebral endothelia as from vessel growth into the embryonic brain, and undergo remarkable modifications in shape, extension, and surface features during the neural angiogenesis and BBB formation (60,61). In the chick embryo optic *tectum*, these cells are irregularly surfaced in the proliferating vessels and cover the 97% of the vessel perimeter when the endothelial cell tight junctions begin to form and the vessel permeability decreases; they are flattened and smooth-surfaced, surrounded by the basal lamina, and almost completely encircled by astrocytes in the mature, barrier-provided vessels (27,61). These findings together with observations that the pericyte investment constitutes a second, defense layer when the endothelial barrier is immature or damaged, and widens when the integrity of the perivascular astrocytic sheath fails, introduce quite rightly the pericytes among the cells having relationship in the BBB system (43,59,62).

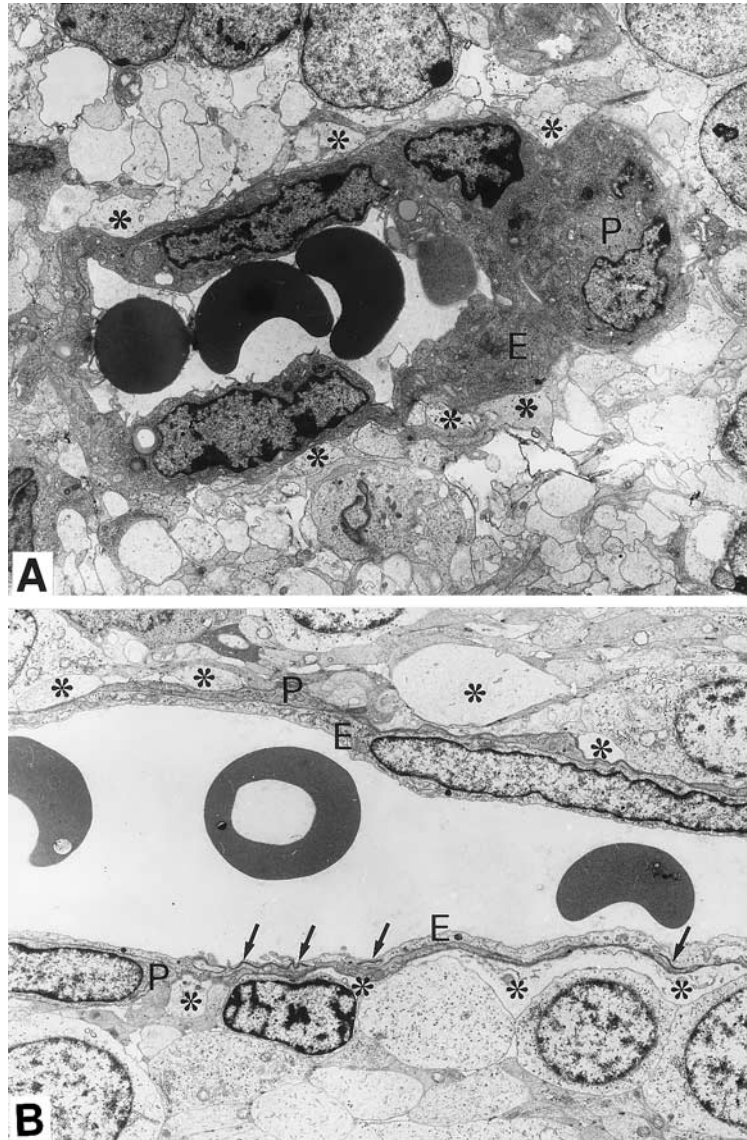


Fig. 1. Electron micrographs of radial microvessels of the human fetus telencephalon at the 12th (**A**) and 18th (**B**) gestational weeks. Differences are detectable between the microvessels in the two developmental stages. In comparison with the 12th wk, the 18th wk microvessel is characterized by reduced thickness and remarkable smoothness of the endothelial cells (E) that are sealed by extensive tight junctions (arrows); there is flattening of the pericyte body and processes (P) that are included in a continuous basal lamina. In both microvessels the endothelium-pericyte layer is closely surrounded by processes of astrocytes (asterisks). (**A**) $\times 6000$; (**B**) $\times 5500$.

In the adult brain microvessels, the basal lamina is thick and continuous under the endothelial cells and splits into two layers surrounding the pericytes interposed between the endothelial layer and the astrocytic perivascular sheath. The various basal lamina components, type IV collagen and noncollagenous glycoproteins such as

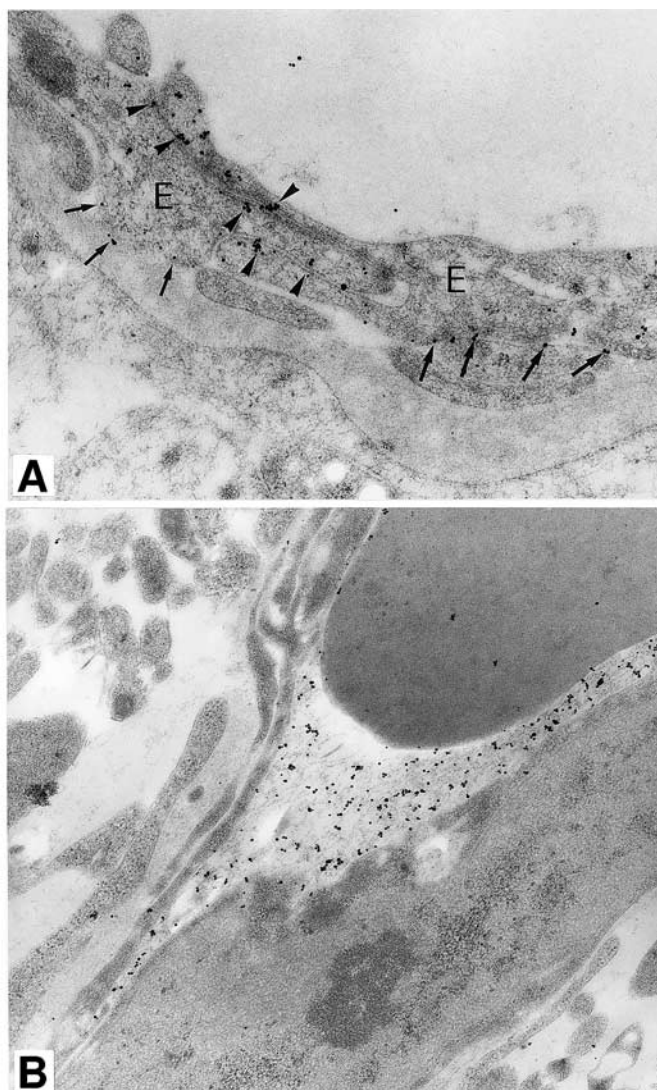


Fig. 2. (A) GLUT1 electron immunohistochemistry of a radial microvessel in the 12 wk human fetus telencephalon. The ends of two endothelial cells (E) welded by a tight junction are labeled by gold particles that are mainly distributed along the abluminal (arrows) and lateral (arrowheads) plasma membranes. (B) Albumin electron immunohistochemistry of a radial microvessel in the 12 wk human fetus telencephalon. The gold particles are localized exclusively inside the vessel lumen. (A) $\times 68000$; (B) $\times 40000$.

fibronectin, laminin, tenascin, as well as proteoglycans, are produced by both vascular cells and perivascular astrocytes and are differently expressed in the neural angiogenesis and BBB development, fibronectin and tenascin being more expressed in early stages (26,63–67). A continuous basal lamina, rich in laminin and glycoconjugates containing β -D galactose residues, is contemporary with the endothelial growth slowing down and BBB maturation (48,68). Despite the difficulties of immunodetection of the molecular components of the developing basal lamina and the discrepancies

between light microscopy immunocytochemistry and electron microscopy observations, previous and recent studies strongly suggest that the basal lamina development is enhanced when and where astroglial processes make contact with the vessel surface and that the basal lamina maturation coincides with the organization of an astroglial perivascular sheath (27,69,70).

3. CHANGES IN THE ASTROCYTIC PERIVASCULAR GLIA

Like the endothelial cells and basal lamina, the astrocytic perivascular glia also undergo remarkable changes during development. Progressively more differentiated types of cells of astroglial lineage establish relationships with the brain vessels. As stated above, their walls are contacted by processes of the radial glial cells at an early stage, and by processes of astroblasts and astrocytes later on. These processes contain both vimentin and glial fibrillary acidic protein cytoskeletal filaments, the vimentin expression prevailing in the early steps of gliogenesis (26,71–73). Further developmental changes undergone by the astrocyte perivascular processes concern the distribution of the intramembrane particles (IMPs) on their joined plasma membranes. The changes consist of the formation of orthogonal assemblies of small intramembrane particles (OAPs) and of gap junctions that, together, characterize the plasma membranes of the astrocytic processes. The OAPs are accumulated in the plasma membrane of the perivascular astrocytic end feet facing the blood vessels and progressively increase in number and size during astrocyte maturation and BBB formation (Fig. 3a, b), presumably playing a physiological role in the water and ion transport in the mature form (74–80). The OAPs are seen to be numerous where the astrocyte plasma membranes are surrounded by a basal lamina, as in the subpial astrocytic processes where these particle assemblies might share in membrane stabilization (81). In the chick embryo brain vessels, the OAP arrangement is time-related to basal lamina formation and BBB differentiation; the possibility that OAPs may have a mechanical function in astrocyte membrane stabilization has therefore to be considered even in the setting up of the BBB. The gap junctions are assemblies of large IMPs that form channels for the diffusion of ions and appear closely packed in hexagonal arrays in the freeze-fractured vessels. In the adult human brain microvasculature, gap junctions are extensively distributed on the plasma membranes of adjacent perivascular astrocytic end feet (82), but gap junctions are also seen by electron microscopy in developing cerebral vessels (Fig. 4a, b). It seems conceivable that the final, ordered alignment of astrocyte end feet close to the microvessel wall in the adult brain may depend on the attraction forces bringing astrocyte processes close together throughout the CNS (83) and is concluded with the establishment of the gap junctional systems between them.

4. GAP JUNCTIONS

The early formation of gap junctions in the microvessels of the prenatal brain is also documented by research carried out in the human fetus telencephalon with connexin 43 (Cx43) immunocytochemistry (Fig. 5a, b, c). Cx43 is a protein component of the gap junction units that constitute the intercellular channels coupling adjacent cells. Unlike other CNS connexins, Cx43 is detectable in the prenatal and postnatal brain and is specifically expressed by both astrocyte precursors (radial glia) and mature astrocytes

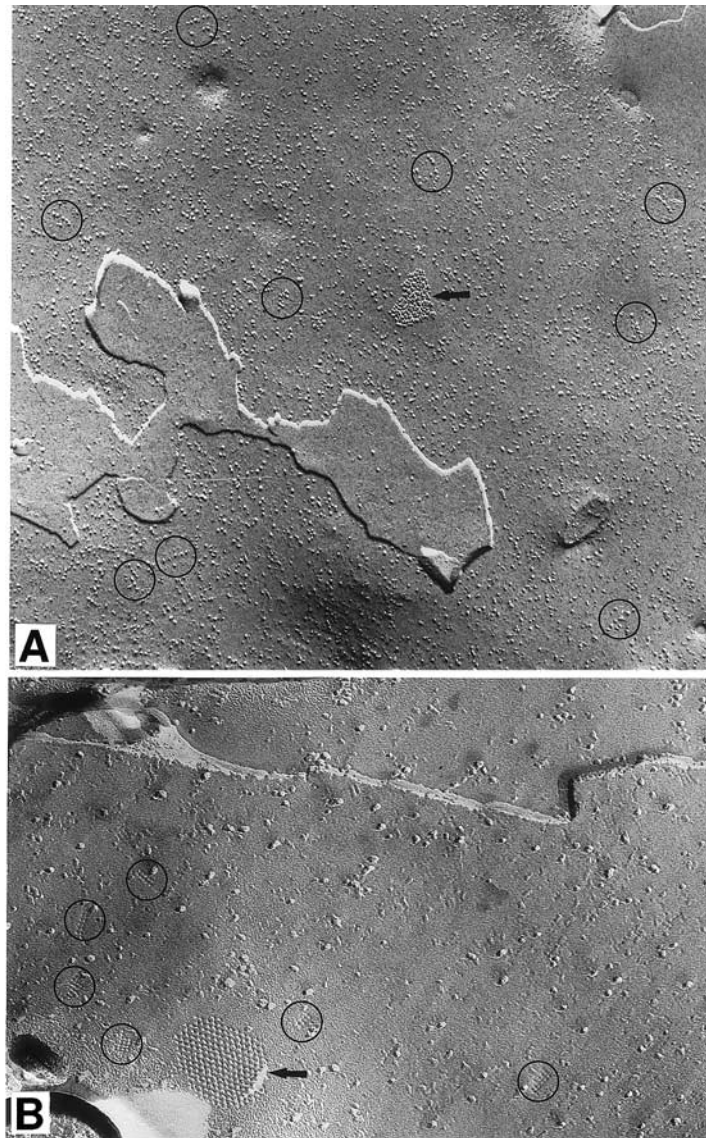


Fig. 3. Replicas from microvessels of 14 d **(A)** and 20 d **(B)** chick embryo optic *tecta* showing plasma membranes of perivascular astrocyte processes. **(A)** Single chains, and short double chains, of aligned individual IMPs (circles) are recognizable, together with a particle cluster identified as a primordial gap junction (arrow), on the P-face of the fractured plasma membrane. **(B)** The astrocytic plasma membrane E-face shows orthogonal assemblies, square or rectangular in shape and of varying sizes (circles); a gap junction plaque is also present (arrow). **(A)** $\times 76000$; **(B)** $\times 155000$.

(84,85). In the human telencephalon, Cx43 labels the radial glial cells and fibers, as well as bipolar and multipolar astroblast perikarya and processes. Cx43 is also detectable in the vascular compartment of the telencephalon when the corticogenesis is under way and radial vessels cross the cortical plate and the subcortical zones coming

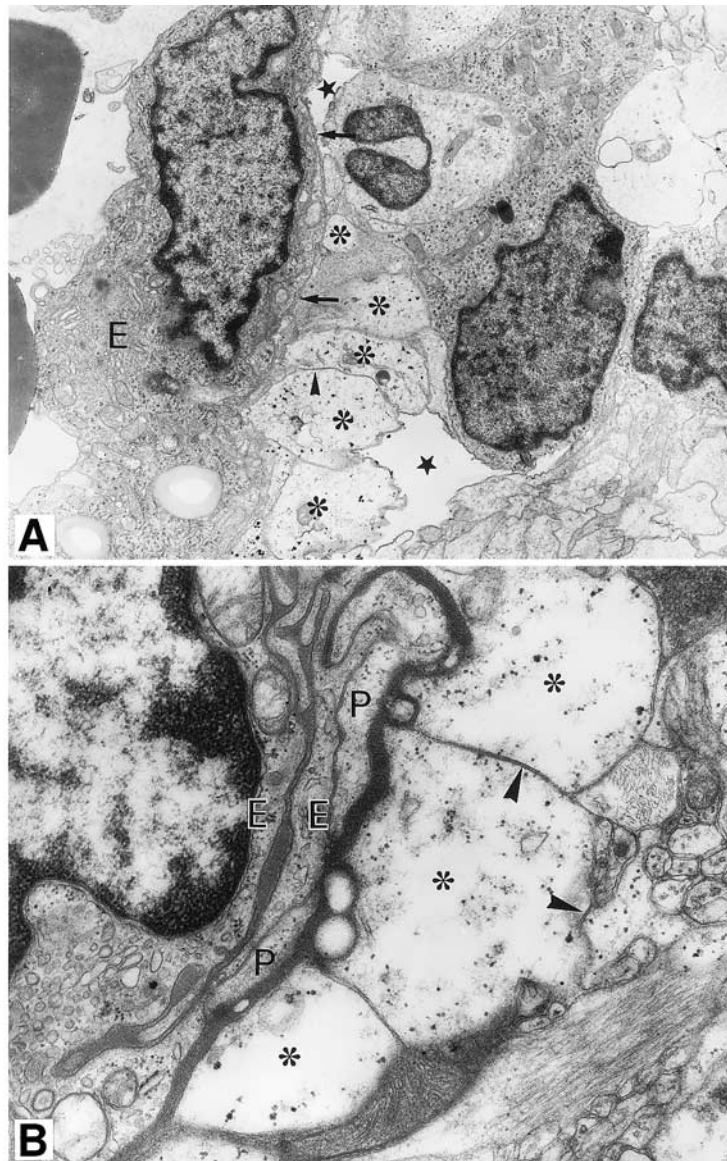


Fig. 4. (A) Electron micrograph of a radial microvessel of the human fetus telencephalon at the 12th gestational week. The abluminal side of a thick endothelial cell (E) is partly surrounded by a basal lamina (arrows) and by perivascular astrocyte processes (asterisks). A gap junction is detectable between adjacent glial end feet (arrowhead). Small spaces are observed in the perivascular tissue (stars). (B) Electron micrograph of a capillary of the adult human cerebral cortex. The thin vessel wall, built by endothelial cells (E) facing a very narrow lumen and by flattened pericytic processes (P) enclosed within the basal lamina, is surrounded by tightly packed astrocyte end feet (asterisks) and a compact neuropil. The arrowheads point to interastrocytic gap junctions. (A) $\times 14000$; (B) $\times 42000$.

together in a plexus located in the ventricular zone. In the ventricular and subcortical zones, the Cx43 immunoreactivity is mainly continuous in the endothelial cells, pericytes and surrounding glia, whereas in the cortical plate the immunoreactivity appears finely punctate (Fig. 5A,B,C) indicating the presence of differentiated vascular gap junctions. Gap junctions between endothelial cells have been already described in the embryonic cerebral microvessels and are transient structures decreasing in number as the endothelial tight junctions differentiate and BBB maturation progresses (86). Gap junctions between endothelial cells and pericytes control the vessel growth processes in the embryonic CNS and persist in the adult brain, where the endothelium-pericyte coupling is involved in the regulation of the blood flow (87,88). The punctate Cx43 immunolabeling shown in the cortical plate of the human telencephalon (Fig. 5c) indicates that gap junctions earlier establish in the wall of microvessels distributing to the most differentiated telencephalic layers. Furthermore, the developmental expression of the same connexin in endothelial cells, pericytes and perivascular astrocytes strongly suggests that communication by gap junction may be involved in the control of the BBB maturation during brain ontogenesis (89).

5. CONCLUSION

In conclusion, many observations of vessel features and properties made in *in situ* studies during the brain embryonic and fetal development seem to indicate that the vascular and perivascular cells undergo differentiating changes and suggest that the BBB progressively matures during prenatal life. In addition, the results demonstrate that processes of astrocyte precursors or mature astrocytes have reached the vessel walls and are arranged close to their endothelium-pericyte layer by the time that the endothelial cells express some signs of morphofunctional tightness and biochemical specificity.

The parallel, prenatal “aging” involving both endothelial cells and perivascular astrocytes strongly substantiates the hypothesis that relationships established between them are involved in setting up the BBB. Such relationships could be mutual and imply not only the widely discussed inducing role that the astrocytes may play on endothelial cell differentiation but also, vice versa, a controlling role exerted by the endothelial cells on the astrocytes (90–92).

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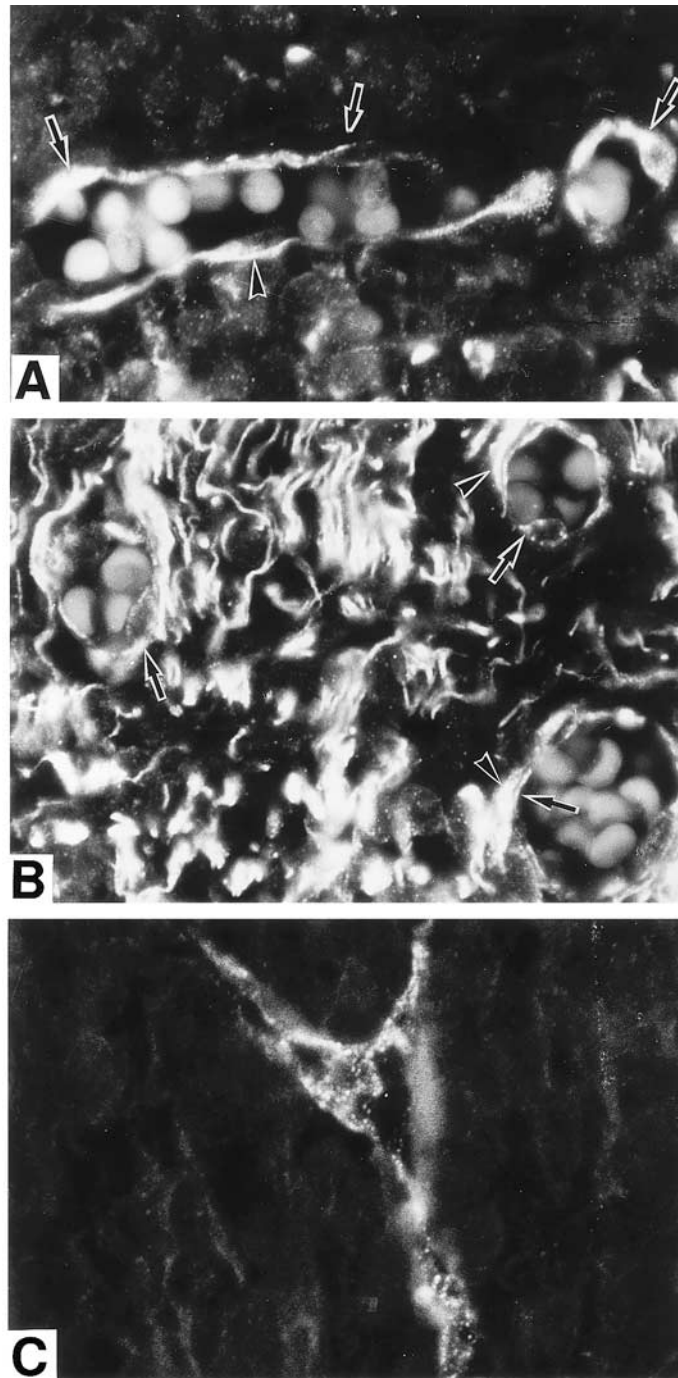


Fig. 5. (A–C) Cx43 immunohistochemistry in the 18 wk human fetus telencephalon. (A) The microvessels of the ventricular plexus show a strong immunoreactivity of endothelial cells (arrows) and subjacent pericytes (arrowhead). (B) In the subcortical zone immunolabeled radial glial fibers are seen to establish close contacts with the vessel wall formed by heavily stained endothelial cells (arrows) and pericytes (arrowheads). (C) A radial vessel of the cortical plate shows a punctate Cx43 immunolabeling. (A,B) $\times 1100$; (C) $\times 1250$.

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VI

ASTROCYTES IN NEURODEGENERATIVE DISEASES

Microglial and Astrocytic Reactions in Alzheimer's Disease

Douglas G. Walker and Thomas G. Beach

1. INTRODUCTION

In the last 20 years, there have been major advances in understanding the causes of Alzheimer's disease (AD) that have allowed the development of different treatment strategies. Progress has been particularly rapid since 1984, with the determination of the amino acid sequence of the amyloid beta ($A\beta$) peptide in cerebrovascular amyloid (1), and plaque cores (2). From this finding, the identification of the amyloid precursor protein (and its gene), (3,4,5) as being the protein from which plaque $A\beta$ is derived, was possible.

There are now known to be gene defects on chromosome 1, 14 or 21 that can lead to early onset forms of AD (6,7,8,). These cases are extremely rare, but have helped in the understanding of the possible mechanisms involved in AD cases that have no identifiable genetic component (possibly 80%). The majority of AD cases can be considered late-onset, with significant incidence only being detectable after age 65. Although AD is primarily a disease of aging, a number of risk factors associated with this disease have been identified. The major one is possession of the apolipoprotein E4 (ApoE4) allele. This allele is possessed by approx 16% of the normal human population, whereas approx 50% of late-onset AD patients possess the ApoE4 allele (9). This finding has been repeated in a large number of subsequent studies with similar results. It does mean however that approx 50% of AD patients *do not* have the ApoE4 allele. Other genetic markers, including one on chromosome 12 have been proposed, but confirmations are still required (10–12). Environmental risk factors that have been linked to AD are head trauma (13), old age (14), and coronary artery and cerebrovascular disease (15). A role for aluminum (16) or viruses (17) has also been proposed, however the evidence has never been convincing.

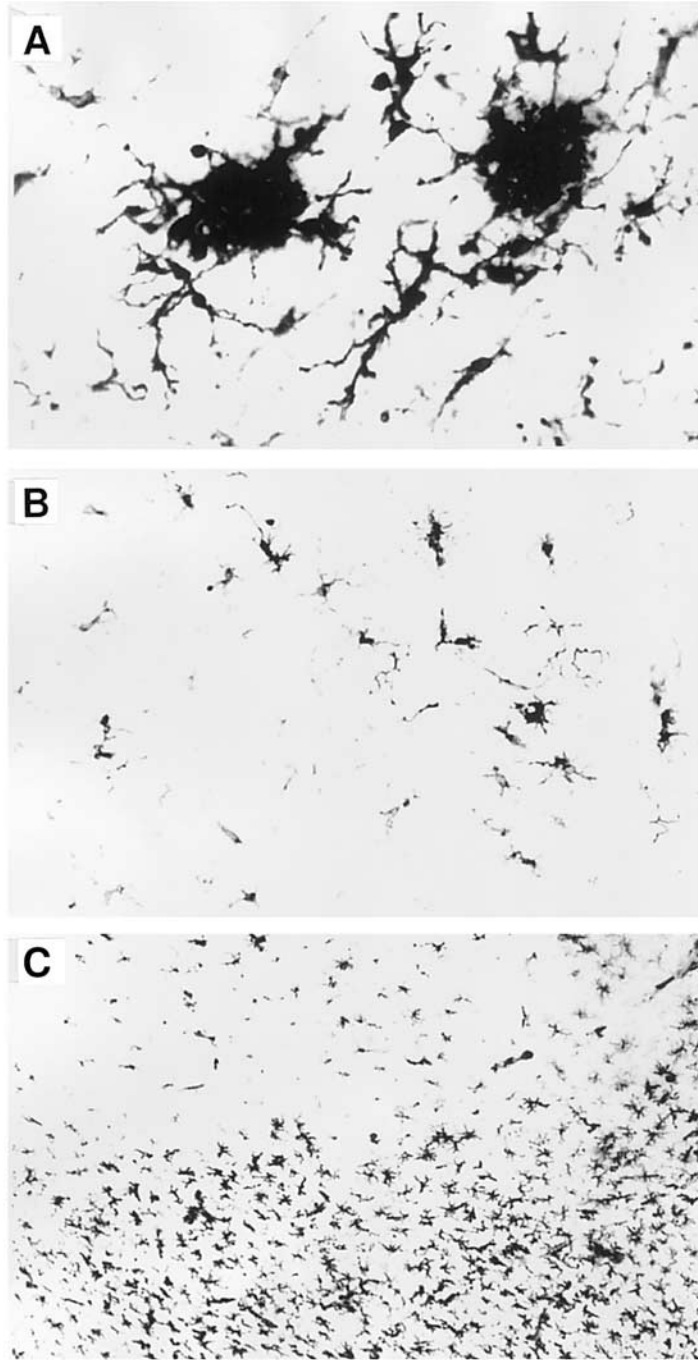
Common pathological features for all types of AD are the development of $A\beta$ -containing plaques, neurofibrillary tangles, dystrophic neurites and also the development of a reactive glial response. The purpose of this article is to consider the possible role of the reactive glial response in the neurodegenerative changes and how it may be involved in the pathogenesis of AD.

2. MICROGLIAL REACTIONS IN AD BRAINS

Microglia are considered to be a population of dendritic cells that have the same origin as monocytes and macrophages, but are normally resident in the brain. In comparison to the other glial cells, the oligodendrocytes and astrocytes, microglia are believed to have migrated into the brain tissue during early brain development, and are not derived from neuroectoderm cells. With the development of modern immunohistochemical techniques and monoclonal antibodies that recognize macrophage proteins, there has been a number of published reports describing the interactions of microglia with the hallmark pathological structures of AD (examples (18–26,68,97,99,117,123,125,144)). However it should be remembered that the early pioneers of neuroscience, including Hortega, (27) and Penfield (28) using classical histochemical staining methods, observed the microglia in the brain, and suggested their potential role in degenerative diseases such as AD.

The typical appearances of microglia that are closely associated with senile plaques are shown in Figure 1A. This section from the temporal cortex of an AD patient was stained with the antibody CR3/43 that recognizes the major histocompatibility complex class II proteins, particularly HLA-DR. In many immunohistochemical studies, this marker has been used to identify activated microglia within gray matter regions of affected brains. In the figure shown (Fig. 1A), the staining identifies a cluster of microglial cells with hypertrophied cell bodies and shortened-enlarged processes, which are located on and around an A β immunoreactive plaque. The morphology of reactive and also resting microglia is shown in figure 1B. This section was stained with an antibody to CD64 that reacts with all microglial cells. The resting cells have a smaller cell body with longer multipolar-ramified processes. The significance of MHC class II expression as a marker of microglial activation is complicated by the observations that robust expression of this marker can be observed on microglia in white matter regions of normal human brain. Figure 1C shows the margin between the white matter region of cortex (lower section) and gray matter (upper section) of an AD case. Although this case had extensive AD neuropathological changes in the gray matter, the strongest HLA-DR immunoreactive microglia were found throughout the white matter. Similarly, in non-AD cases, HLA-DR immunoreactive microglia are also found throughout the white matter (21,29). Although the significance of increased microglial

Fig. 1. (Right) (A). Photomicrograph showing highly reactive microglia clustered on and around A β immunoreactive plaques. The section is from the inferior temporal gyrus of an AD case. The section was stained with the antibody CR3/43 that recognizes the MHC class II protein HLA-DR. **(B).** Photomicrograph showing a range of different morphologies of microglia in a section from an AD case. The section was stained with an antibody to CD64 (Fc γ RI). This antibody reacts with all microglia, but more strongly with those with a reactive morphology. **(C).** Photomicrograph showing the strong HLA-DR immunoreactivity of microglia in white matter. The section is from an AD case and shows the boundary between white matter (lower section) and gray matter (upper section). In the gray matter, the microglia are more associated with pathological structures, while in the white matter the staining is more diffuse and not apparently associated with pathology. In normal cases, HLA-DR immunoreactive microglia can be observed in white matter, but generally not in gray matter.



HLA-DR immunoreactivity in relation to defining their “activated” state still remains to be determined, it has proven to be the best marker for identifying activated microglia in areas where degenerative pathology exists, particularly in gray matter regions of the brain. The difference in reactivity between microglia cells in different brain regions

could suggest that they represent different populations of cells. A detailed comparison of the HLA-DR immunoreactive microglia in different brain regions, including white matter, revealed a number of different cellular morphologies (29). However, there was not a noticeable difference in the morphology of white matter compared to gray matter microglia in the series of sections studied (Fig. 1C), with the exception of the microglia associated with the A β plaques and tangles. Although highly immunoreactive, the white matter microglia generally had a ramified (unactivated) morphology. It has been shown that two key enzymes that may be involved in the processing of A β peptide, the membrane-type matrix metalloprotease and gelatinase A, were only detectable in white matter microglia in the brain tissues studied (30,31). These data therefore also suggest that white and gray matter microglia may represent different populations of cells. Another possibility is that microglia in gray matter regions of brain are exposed to agents that inhibit HLA-DR expression. One such agent may be the colony stimulating factor CSF-1 (32).

There have been several studies in which the relative numbers of microglia, or activated microglia, were quantified in AD and young and elderly control brains. Comparing these studies is complicated by the fact that different markers for microglia were used and different brain regions examined. A significant increase in total number of HLA-DR immunoreactive microglia was quantified in the middle temporal gyrus of AD cases relative to control cases, however these authors did not attempt to correlate the distribution of activated microglia with the presence of plaques or tangles (33). Mackenzie et al. (34) quantified the total numbers of microglia in elderly cases with no plaques, with diffuse plaques, with diffuse and neuritic plaques, and with AD. This study showed that there were increased total numbers of microglia in AD cases, and in the cases with diffuse and neuritic cases in comparison with the cases with no plaques. Also, there was no significant difference in the number of microglia between the cases with no plaques and the cases with only diffuse plaques (34). A similar study of young, old and AD cases, that used an antibody to ferritin to identify activated microglia, showed a significant increase in numbers of microglia counted in eight regions of the hippocampus (35). These authors did not find a significant correlation of the localization of microglia with either senile plaques or neurofibrillary tangles, except for tangles in the subiculum. This finding was generally confirmed in another study, which found that microglial cell distribution correlated poorly with plaque distribution in the dentate gyrus of AD cases (36). In trying to understand the significance of microglial MHC class II protein expression and microglial activation in AD pathology, it has generally been observed that microglia around diffuse amyloid deposits are not expressing increased amounts of MHC class II. Immunohistochemical staining with an antibody to HLA-DR of sections of entorhinal cortex and inferior temporal gyrus from the brains of a series of nondemented young and elderly cases confirmed this observation (Schwab et al., unpublished observations). There was a significant increase in numbers of HLA-DR immunoreactive microglia when comparing the elderly with the young cases. However, there was no significant difference in numbers of HLA-DR reactive microglia between the elderly cases without A β plaques and those that did have plaques. These cases primarily had diffuse plaques in the regions studied (Schwab et al., unpublished observations). This finding generally confirms the observations of others where there was not increased HLA-DR immunoreactivity associated with diffuse

plaques in AD cases, in the cerebellum of control or AD cases, or diffuse A β plaques of cases of hereditary cerebral hemorrhage with amyloidosis (Dutch) (20,21,23,26,37). Fukumoto et al. (38) have refined these observations with the use of antibodies that can distinguish between A β plaques ending at amino acid 40 and plaques ending at amino acid 42. A significantly higher proportion of A β -40 plaques were associated with activated microglia than A β -42 plaques. These results were similar in the AD cases and nondemented elderly cases. This result has been interpreted as evidence that the microglia around the plaques are processing the A β -42 to the A β -40 form, however it might also be interpreted to indicate that the A β -42 is deposited earlier than A β -40, and prior to microglial activation.

A series of studies by Griffin and coworkers have indicated that microglia around diffuse plaques may also be activated. In contrast to most studies, these authors have used increased expression of the cytokine interleukin 1 α (IL-1 α) as the marker for microglial activation (57,131,134,136). IL-1 α is a potent proinflammatory cytokine that is induced in macrophage cells by a range of proinflammatory stimuli. These authors observed the highest numbers of IL-1 α immunoreactive microglia associated with uncored neuritic plaques (39). In vitro studies have shown that the activation signals that cause induction of IL1 α are not the same as those that activate the transcription of the MHC class II genes (27,32).

A similar type of microglia response to A β has been observed in the brains of transgenic mice carrying copies of the human APP gene with AD-associated mutations, or rats whose brains have been infused with preparations of synthetic A β . Some of these animals develop AD-like neuritic plaques which have associated reactive microglia and astrocytes (40–43). Evidence that the microglial reaction is in response to the A β plaques was shown by the fact that it was more pronounced in the transgenic mice that had the higher burden of amyloid plaques (43).

There is still some controversy relating to whether there is proof that the activated microglia are contributing to the AD pathology or whether the observed activation is an epiphenomenon. Although in vitro studies indicate the potential for microglia to produce neurotoxic products, there is only indirect evidence that this is occurring in vitro. Morphometric studies that counted the number and distribution of activated microglia in AD and aging brains showed that there was not significant correlation between microglial cell density and plaque or tangle density (35,36). Both of those studies utilized ferritin as the marker to identify activated microglia. Although, as shown, the most activated microglia appear to be arranged around dense A β plaques, overall the pattern of activation is quite diffuse throughout the neuropil of affected brain regions. This might indicate that the insoluble A β or tangles were not the primary activation signals. Microglial activation may also be in response to neuropil threads, synaptic loss, neurofibrillary tangle formation, axonal degeneration or other factors. Many studies have shown that microglial activation is significantly associated with neuritic plaques. It can thus be suggested that some soluble proteins released from damaged neurons may be the key factors involved in microglial activation (44).

3. INCREASED ASTROCYTOSIS IN AD

It has been established that there are increased numbers of glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes in the AD cerebral cortex. A qualitative report

Table 1
Biologically active compounds secreted
by astrocytes and associated with AD pathogenesis

<i>Cytokines</i>
Interleukin 6 (166)
GM-CSF (58)
MCSF-1 (32)
<i>Complement proteins</i>
Complement C3 (167)
Complement C4 (168)
Complement C9 (168)
<i>Complement inhibitor proteins</i>
C1-inhibitor (169,170)
Clusterin (169)
Vitronectin (171)
<i>Protease/Protease inhibitors</i>
α -1 antichymotrypsin (173)
α -2-macroglobulin (174)
<i>Lipid transport molecules</i>
Apolipoprotein E (175)
Apolipoprotein J (clusterin) (169)
<i>Free radicals</i>
Nitric oxide (Nitric oxide synthase) (93)

by Duffy et al. (45) was followed by quantitative work by Schechter et al. (46) and Mancardi et al. (47), which demonstrated fourfold or greater increases in GFAP-immunoreactive astrocytes. Mapping of the pattern of cerebral GFAP immunoreactivity in AD cases (48) found all cortical areas to be consistently and uniformly affected, with subcortical nuclei being frequently but inconsistently involved.

One of the established concepts that requires discussion is the idea that reactive astrocytes are playing a supportive role in trying to help “unhealthy” neurons to survive (49). It has been documented that reactive astrocytes can express a range of neurotrophic agents including nerve growth factor (NGF), acidic and basic fibroblast growth factor (FGF), neurotrophin (NT)-3, brain-derived neurotrophic factor (BDNF), midkine and glial cell line-derived neurotrophic factor (GDNF) (50–54). In addition, astrocytes have been shown to produce a range of antioxidants, protease, and complement inhibitors (see Table 1). However, evidence now exists that astrocytes could be contributing directly or indirectly to the pathological process in AD. This includes the possible production of neurotoxic substances, inhibition of microglial phagocytosis of amyloid plaques (55,56) and the production of pathological chaperone proteins (57).

Staining of human AD tissue sections with an antibody to the glial fibrillary acidic protein (GFAP), the most widely used marker to identify astrocytes, shows that the most reactive astrocytes are arranged around the compact A β plaques (Fig. 2A and 2B).

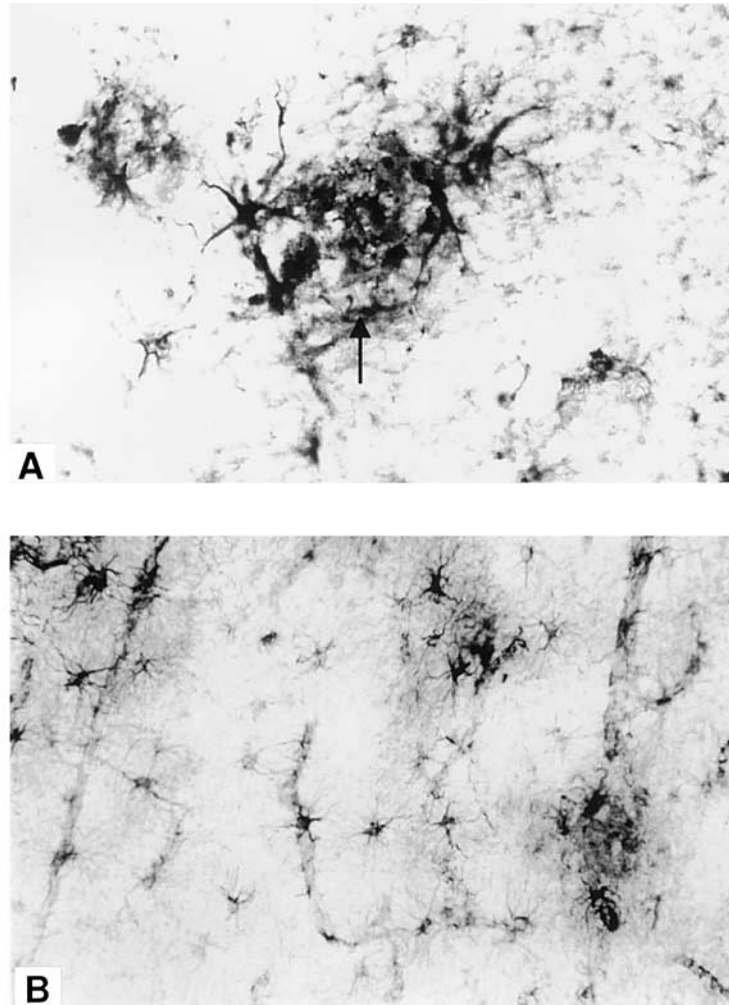


Fig. 2. (A). Photomicrograph of reactive astrocytes clustered around an A β -immunoreactive plaque (arrow). The section from an AD case was immunostained with an antibody to glial fibrillary acidic protein. In comparison to the microglia illustrated in Fig. 1A, reactive astrocytes tend to be found around the periphery of plaques. (B). Lower magnification photomicrograph of reactive astrocytes in the gray matter of an AD case. The immunoreactive cells are found associated with AD pathological structures. In human brains, gray matter astrocytes with a resting morphology exhibit weak or undetectable GFAP immunoreactivity.

This is in contrast to the clusters of highly reactive microglia that tend to be located on the plaque core (Fig. 1A). In the latter case it can be suggested that the microglia are attempting to phagocytose the extracellular A β peptide, however the function of the astrocytes in this process is not clear. Similar to what can be observed for HLA-DR in normal brain, there is stronger GFAP immunoreactivity in the white matter brain regions than the gray matter. One unique feature about the glial reaction in the AD brain may be the cooperation between the highly activated microglia and astrocytes located around the plaque. This could be exacerbating the glial response. Microglia

appear to be the major source of the proinflammatory cytokine IL-1 β , which can induce a reactive phenotype in astrocytes (49). Similarly, IL-1 β can induce astrocyte expression of granulocyte-monocyte colony stimulating factor (GM-CSF). This growth factor is the most effective in inducing proliferation of fetal and adult human microglia (58). GM-CSF immunoreactive astrocytes can be detected around senile plaques in AD brains (58). Astrocytes are also the major source of colony stimulating factor-1, another growth factor for human microglia (58).

4. MARKERS OF MICROGLIAL ACTIVATION

There have been a number of studies describing the expression of different markers (besides HLA-DR) on microglia in AD brains. These are summarized in Table 2. Most of these markers are expressed constitutively by microglia in the resting state, and upregulated around AD pathology. However, one particular marker of interest is melanotransferrin (p97), an iron transport protein. This protein is constitutively expressed by vascular endothelial cells, but is also selectively expressed by a small subset of microglia in association with A β plaques (59,60). Significantly elevated serum concentrations of this protein have been measured in AD cases compared to control cases, and it has been proposed as a potential diagnostic test for AD (61). A number of other studies have shown that ferritin, an iron binding protein, is also a good marker for identifying microglia and under suitable conditions can be used to identify reactive microglia (35,38). Microglial immunoreactivity for ferritin appears not to be as restricted as that observed for melanotransferrin.

Another potential marker for microglial activation is immunoreactivity with antibodies that recognize phosphotyrosine-modified proteins. There has been limited application of this marker for histological studies (40,43), with only one report of results showing phosphotyrosine immunoreactive microglia in human brain tissue from AD cases (62). Although many cells possess tyrosine kinases, antibodies to phosphotyrosine tend to recognize activated microglia in tissue sections. The usefulness of this marker has not been fully investigated, however many *in vitro* studies with cultured macrophages or microglia show that increased tyrosine phosphorylation occurs rapidly as a consequence of many different stimuli that activate tyrosine kinases (63,64). These studies generally showed that tyrosine phosphorylation was short lived as a result of the action of different cellular phosphatases. Immunoreactivity for phosphotyrosine would thus appear to identify microglia that were acutely activated at the time of death. It will be of interest to compare the correlation between microglial expression of HLA-DR and phosphotyrosine.

5. MARKERS OF ASTROCYTIC ACTIVATION

GFAP is a 57 kD intermediate filament protein that is only expressed by astrocytes. In the normal human brain, astrocytes in gray matter regions show weak or undetectable GFAP immunoreactivity. A noticeable increase in GFAP immunoreactivity becomes apparent in areas of degeneration or injury, along with an increase in the size of the astrocyte cell body and its processes. Similar to what has been described for HLA-DR, strong GFAP immunoreactivity is observable in white matter areas of human brains lacking pathology. The significance of increased GFAP expression by astrocytes

Table 2
Expression of markers on microglia in Alzheimer's Disease

Marker	Function	Expression in AD (ref)
MHC class I	Antigen presentation to CD8 positive T cells	Increased (120)
MHC class II	Antigen presentation to CD4 positive cells	Increased (22)
CD11a (LFA-1)	ICAM-1 and ICAM-11 receptor	Increased (121,122)
CD11b (CR3)	Complement C3bi receptor	Increased (121,122)
CD11c (CR4/p150)	Complement C3bi receptor	Increased (121,122)
CD18 (β 2 integrin)	Subunit of CD11 complex	As CD11a, b, c
CD25 (Tac)	Interleukin-2 receptor	Increased (22)
CDw32 (Fc γ RII)	IgG Fc fragment receptor	Increased (121)
CD43 (leukosialin)	Antiadhesion molecule	Decreased (123)
CD45 (LCA)	Receptor with phosphotyrosine phosphatase activity	Increased (124) CD45RO
CD51 (α V integrin)	Vitronectin receptor	Increased (122,125)
CD64 (Fc γ RI)	IgG Fc fragment receptor	Increased (121)
CD68 (macrosialin)	Lysosome-associated membrane protein	Increased (126)
Interleukin-1 α	Proinflammatory cytokine	Increased (39)
Interleukin-1 β	Proinflammatory cytokine	Increased (127)
Interferon- α	Pro/anti-inflammatory cytokine	Increased (128)
Interferon- α receptor	Interferon- α receptor	Increased (129)
Monocyte chemoattractant protein-1	Chemotactic cytokine	Increased (130)
Transforming growth factor- β 2	Anti-inflammatory cytokine	Increased (28)
Tumor necrosis factor α	Proinflammatory cytokine	Increased (127)
MCSF-receptor (c-fms)	Colony stimulating factor receptor	Increased (131)
Ferritin	Iron transport protein	Increased (38)
Melanotransferrin	Iron transport protein	Increased (59)
Phosphotyrosine-modified proteins	Product of tyrosine kinase action	Increased (132)
Macrophage scavenger receptor	Lipoprotein receptor	Increased (133,134)
VLDL-receptor	Lipoprotein receptor	Increased (136)
Vimentin	Cytoskeletal protein	Increased (137)
Plasminogen activator inhibitor	Serpin protease inhibitor	Increased (138)
Factor XIIIa	Coagulation factor	Increased (139)
Bcl-xl	Apoptosis inhibitor	Increased (140)
Tissue factor pathway inhibitor-1	Protease inhibitor	Increased (141)
Myeloperoxidase	Enzymes, generates free radicals	Increased (142)
CCR3, CCR5	Chemokine receptors	Increased (143)
C/EBP α	Transcription factor	Increased (144)

Table 3
Expression of markers on astrocytes in Alzheimer's Disease

Marker	Function	Expression in AD (ref)
GFAP	Intermediate filament	Increased (48)
S100 β	calcium binding protein	Increased (69)
TGF- β 2	Anti-inflammatory cytokine	Increased (145)
Heat shock protein 27	Stress protein	Increased (146)
Basis fibroblast growth factor	Neurotrophic agent	Increased (53)
Acidic fibroblast growth factor	Neurotrophic agent	Increased (54)
Fibroblast growth factor-9	Neurotrophic agent	Increased (147)
FGF receptor 1	Fibroblast growth factor receptor	Increased (148)
TrkA receptor	Nerve growth factor receptor	Increased (149)
TrkB receptor	Nerve growth factor receptor	Increased (150)
MIP-1 β	Chemokine	Increased (143)
Hepatocyte growth factor	Growth factor	Increased (151,152)
Metallothioneins I and II		Increased (153)
α -1 antichymotrypsin	Protease inhibitor	Increased (154)
Apolipoprotein E	Lipid transport protein	Increased (37)
Apolipoprotein J (clusterin)	Lipid transport protein	Increased (155)
PP-2A, PP-2B	Protein phosphatases	Increased (156)
Insulin growth factor-1	Growth factor	Increased (157)
Manganese superoxide dismutase	Free radical scavenger	Increased (158)
Bcl-x1	Apoptosis inhibitor	Increased (140)
Cytosolic phospholipase A2	Phospholipase	Increased (159)
Fas	Cell surface apoptosis initiator	Increased (160)
Nitric oxide synthase	Enzyme	Increased (95)
CD44	Receptor/adhesion molecule	Increased (161)
Intercellular adhesion molecule-1	Adhesion molecule	Increased (162)
Vimentin	Intermediate filament	Increased (137)
Vasoconstrictor endothelin-1	Vasoconstrictor peptide	Increased (163)

as they develop a reactive profile remains to be determined. There have been conflicting reports on this matter. Treatment of cultured astrocytes with antisense sequences of GFAP prevented them from developing a reactive profile (65–67). However, on the other hand, mice with a deletion of the GFAP gene were able to develop a normal reactive gliosis response to an experimental insult (66). However, a recent study showed that astrocytes from mice lacking expression of GFAP could not develop a reactive response to A β (68).

Other markers for activated astrocytes have been described in AD brains. These are listed in Table 3. In contrast to GFAP, none of these are specific to astrocytes, but are more highly expressed by these cells compared to others.

Of the listed markers, S100 β has been especially interesting as a marker for activated astrocytes. S100 β is a calcium-binding protein secreted by glial cells in the central and peripheral nervous systems. S100 β promotes neuronal differentiation and survival but may be detrimental to cells if overexpressed. It is also a mitogen for glial

cells. The overexpression of S100 β in AD brains relative to control cases, correlated well with the pattern of regional involvement by neuritic plaques, with the largest increases being in the hippocampus and temporal cortex, and the smallest in the cerebellum (19,69). S100 β was shown to increase APP mRNA in neuronal cultures, and A β peptide increased S100 β expression in astrocyte cultures (70–73). It has been suggested that neurite-promoting properties of S100 β may contribute to the formation of dystrophic neurites associated with certain classes of A β plaques (74). The expression of S100 β by astrocytes was increased by the cytokines IL-1 α and IL-1 β (75.) These data confirm the probable interaction between microglia and astrocytes contributing to AD neuropathology.

6. CONTRIBUTION OF GLIA TO AD PATHOLOGY

There has been much speculation as to the contribution of reactive glia to the formation of AD type pathology. Some researchers believe that it is the interaction of these glial cells with the A β peptide, rather than the direct toxic effect of A β on neurons, that is causing much of the AD neuropathology. There has been a range of glial-derived toxic agents suggested, including nitric oxide, reactive oxygen species, cytokines, glutamate, phenolic amine like compound and peptides (76–84). We will not try and discuss them all, but concentrate on discoveries where the in vitro data is backed up with in vivo observations.

7. MICROGLIA-DERIVED NEUROTOXINS

Of particular note has been the observations on the production by cultured microglia of a putative small molecule phenolic-amine like toxin (designated NTox), when these cells interact with A β derived from plaque cores purified from AD brains, or synthetic A β peptides corresponding to the human, but not rodent, sequences (85,86). This compound has not been fully characterized chemically, but is specifically induced by microglia upon interaction with A β peptides containing the 10–16 domain sequence and the sequences at the C terminal of the peptide (ending in 40 or 42) (86). The data suggest that the A β region 13–16 is required for binding of the peptide to a putative microglia receptor, whereas the C-terminal sequence is necessary for the induction of the NTox. NMDA receptor antagonists could block the action of this toxin on neuronal cells (86). The induction of toxin by microglia could be blocked by treatment of cells with the peptide HHQK, which corresponds to A β amino acids 13–16 (87). Using synthetic A β peptides, there did not appear to be a significant difference between A β 1–40 and A β 1–42 in their efficacy in inducing the production of this toxin. This toxin was produced in a similar manner by A β -stimulated human or rodent microglia. A toxin that was isolated directly from AD brains behaved in the same neurotoxic manner as the toxin produced by A β -stimulated microglia (85).

8. ASTROCYTE-DERIVED NEUROTOXINS

A possible involvement of toxic forms of nitric oxide (NO) in AD has been suggested by the demonstration of increased amounts of nitrated residues of tyrosine in neurofibrillary tangles, and in neurons showing evidence of DNA damage (88,89). The presence of nitrotyrosine-modified proteins is direct evidence for the action of peroxynitrite. Increased production of NO, along with its interaction with superoxide, can

result in the formation of peroxynitrite, a highly reactive product. This can oxidize proteins resulting in the formation of carbonyl, as well as modify tyrosine to form nitrotyrosine. Nitrotyrosine can be measured by high performance liquid chromatography techniques, along with electrochemical detection, whereas it can be localized by immunohistochemistry with nitrotyrosine specific antibodies (90).

There are three different nitric oxide synthase (NOS) enzymes present in different cells of the brain. NOS type I is found in neurons, NOS type III is found mainly in endothelial cells, whereas NOS type II (also called inducible NOS) is induced by astrocytes and microglia in response to inflammatory stimuli. It is thus possible that the increased synthesis of NO could be derived from cells other than glia, however activated glial cells can produce large amounts of NO relative to neurons or endothelial cells. It has been demonstrated that microglia from rodents can be stimulated to produce large amount of NO as a result of stimulation of expression of the inducible NOS gene. However it appears that human microglia do not readily express inducible NOS (26,64,80), though this is still a subject of controversy (91).. Human astrocytes can be stimulated by inflammatory cytokines to produce NO (92,93). NO derived from activated astrocytes can be neurotoxic to human neurons (77,94). In vitro studies have shown that the astrocytic produced NO can induce apoptosis in cocultured neurons. The neurotoxic effect can be blocked by coincubation of stimulated astrocytes with a NOS inhibitor (77,94). Astrocytes expressing inducible NO synthase have been observed associated with plaques in AD brains, using enzyme and immunohistochemistry techniques (95). This in vivo observation provides evidence for the possible contribution of astrocyte derived NO to AD pathogenesis.

9. ASTROCYTE-DERIVED PATHOLOGICAL CHAPERONE PROTEINS

The contribution of the amyloid-associated proteins to the pathogenesis of the disease has been intensely studied. A large number of proteins have been shown to become associated with A β plaques and neurofibrillary tangles. Of particular interest are the complement proteins. The potential role of these in AD have been extensively reviewed (96,97,98).

The possible microglial and astrocytic origins of some of these are listed in Tables 1 and 4. The proteins that have been most intensively studied have been apolipoprotein E (apoE) (57), α -1-antichymotrypsin (99), heparan sulfate proteoglycan (100), apolipoprotein J (apoJ-clusterin) (101), and complement C1q (102). These compounds are of particular interest as they have been shown to modulate the rate of formation of A β peptide into a beta-pleated sheet configuration. A consequence of this would be either to transform soluble (diffuse) A β to the toxic fibril form, or to stabilize the plaques and tangles thus rendering them more resistant to degradation. ApoJ is also associated with A β plaques, however binding of this protein to A β appears to inhibit fibril formation (101). Some studies have suggested that apoJ-A β complexes are also neurotoxic (103). With the exception of C1q, which is derived from microglia or neurons, the other components are primarily derived from astrocytes. In vivo studies have demonstrated immunoreactivity or mRNA for apo E, apo J, α -1-antichymotrypsin and heparan sulfate proteoglycan in astrocytes, with increased expression being detectable in those cells closely associated with plaques.

Table 4
Biologically active compounds produced
by microglia and associated with AD pathogenesis

<i>Cytokines (107)</i>
Interleukin-1 α
Interleukin-1 β
Interleukin-6
Tumor necrosis factor α
<i>Complement proteins (107,126)</i>
Complement C1q
Complement C3
Complement C4
<i>Complement inhibitor proteins</i>
C1-inhibitor (164)
C4 binding protein
<i>Free radicals/small molecules</i>
Reactive oxygen species (165)
NTox (phenolic amine) (86)
Glutamate (80,81)
Quinolinic acid (48)

It can be suggested that the astrocytic response in AD that appears to develop as a consequence of the microgliosis is likely to have a detrimental effect on the pathological process, rather than a reparative response.

10. MODELING GLIAL RESPONSES IN ALZHEIMER'S DISEASE

A number of research groups are discovering that glial cells derived from post-mortem or biopsy samples of human brains can be prepared and used in experiments (104,85,105,106,107,108). Techniques based on those developed for isolating human oligodendrocytes have proven suitable for preparing pure cultures of microglia from postmortem brains, as well as human biopsy material (109). The availability of such cells is dependent on being able to obtain brain tissue from cases as soon after death as possible (ideally less than 4 h) (106,107). In a series of glial cell isolations, using white matter tissue from cases that were obtained less than 5 h after death, we were able to obtain 3.02×10^6 viable cells/gram of tissue (standard deviation of 1.69×10^6 , n=25 cases). In comparison to microglia derived from fetal brains, postmortem brain-derived microglia do not readily replicate so the numbers available for experimentation, even if tissue is available, remains limited. Their responses to stimuli may more accurately reflect microglial reactivity in elderly human brains than do those from fetal microglia. Differences between adult and fetal microglia have been identified, as have differences between microglia derived from young and aged rats. It was observed that human fetal microglia and astrocytes were more responsive to proinflammatory stimuli than were adult brain-derived glial cells (105).

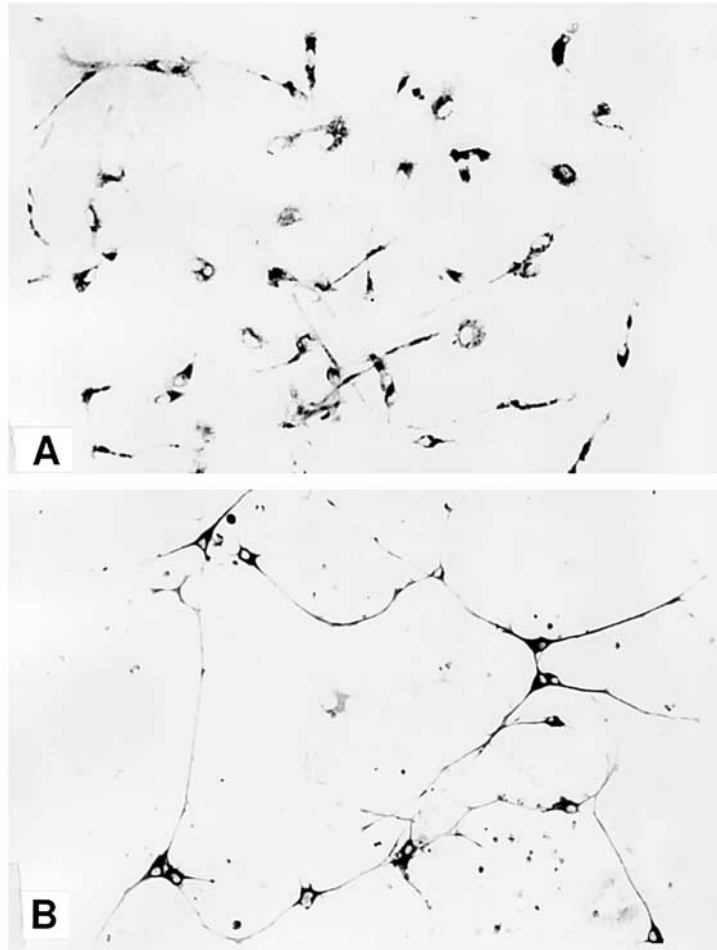


Fig. 3. (A). Photomicrograph of microglia derived from human postmortem brain tissue. The cells were cultured *in vitro* for three weeks after death prior to use in experiments. The cells were identified using an antibody that recognizes the macrophage specific marker CD68 (macrosialin). This protein is located on lysosomal membranes. Reactive microglia that have phagocytosed material show stronger CD68 immunoreactivity. **(B).** Photomicrograph of fibrous astrocytes-derived from human postmortem brain tissue. The cells were cultured *in vitro* for four weeks after death prior to use in experiments. These cells are separated from microglia. Cells were identified by immunostaining with an antibody to GFAP. These types of cells can be maintained up to eight weeks in culture. They do not exhibit evidence of cell division. In similar cultures, protoplasmic astrocytes can also be seen. These cells will divide slowly.

In contrast, microglia cultured from elderly rats appeared to have a higher degree of spontaneous reactivity, as measured by MHC class II expression, compared to cells from young rats (110). In addition, the microglia from elderly rats appeared less responsive to the antiinflammatory effects of transforming growth factor β (110).

Examples of glial cells isolated and grown from human postmortem brains are shown in Fig. 3.

11. DISCUSSION

There has been tremendous scientific investigation into the glial responses that are occurring in AD, and how they may be involved in the pathology of AD. It is possible to list all the potentially damaging products of these glial cells, and assume that they are doing damage in the human brain, because this can be demonstrated in isolated cell culture systems. However there is also evidence for increased expression of antiinflammatory cytokines, antioxidants, and protease and complement inhibitors by these same glial cells. In particular, it is likely that false conclusions will be drawn if emphasis is placed on the role of only one or two of these glial products. As shown there are now a large number of these products that interact with microglia, astrocytes, plaques or tangles. As AD tends to be a chronic disease, with the affected showing gradual cognitive decline, it can be suggested that the inhibitory proteins may gradually be overcome, leading to synaptic loss, cell death, and dementia.

Final interpretation of all the data, both from pathological studies and in vitro model system, indicate that it has not been definitively proven that glial cells are contributing to the pathology of AD, though the data strongly suggests this conclusion. It has been suggested that proof of microglial involvement in AD has come from the findings that antiinflammatory drugs are effective at slowing the progression of AD (111). The problem with this interpretation is that the effective drugs share the common property of being cyclooxygenase (COX) inhibitors (111). There are two different cyclooxygenases. COX-1 (constitutive cyclooxygenase) and COX-2 (inducible cyclooxygenase) exist in different cell populations. The effective drugs tend to have greater activity as COX-1 inhibitors, however, they can also inhibit COX-2 at higher concentrations. In the brain, COX-2 is readily detectable, although COX-1 is present at much lower abundance (112). Also of interest is the fact that different populations of neurons express high levels of COX-2, whereas microglial expression of this protein has not been definitively demonstrated in vivo in the human brain (110,113). Thus the "antiinflammatory" drugs may be acting directly on vulnerable neurons, not on the inflammatory associated cells (114). Model systems for ischemia have shown that COX-2 is increased in neurons following toxic insult (115,116). This increased neuronal COX-2 expression can lead directly to cell death.

Similarly, propentofylline has been proposed to be effective in AD (117) because of its antiinflammatory properties, possibly by inhibiting microglial superoxide production (118). However this agent, an adenosine reuptake inhibitor, is also effective in stimulating increased nerve growth factor secretion by astrocytes (119).

Considerable progress has been made in the last decade to describe the glial response that is occurring in the brains of AD cases. These observations have been combined with a range of experimental model systems that describe mechanisms that might be occurring in the AD brain. Continued detailed studies are required to confirm that inhibiting the glial response will be beneficial to the AD patient. This will be aided by the availability of agents that are specific in inhibiting the glial-associated response.

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Activated Neuroglia in Alzheimer's Disease

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1. INTRODUCTION

The distinguishing pathological hallmark of Alzheimer's disease (AD) is the presence of proteinaceous deposits, referred to as senile plaques, within the higher learning centers of the brain (1). These plaques are comprised predominantly of multimeric fibrils of the amyloid β ($A\beta$) peptide (2), a 40–43 amino acid long proteolytic fragment that is cleaved from the larger amyloid precursor protein (APP) (3). Genetic data suggest that senile plaques are key causative agents in AD, as all known mutations that cause early-onset familial AD (4–8) result in an increased production of the amyloidogenic $A\beta_{1-42}$ isoform (9–13). Although the deposition of multimeric $A\beta$ fibrils into plaques is likely to be a requisite step in AD onset, there is still uncertainty as to how $A\beta$ and neuritic plaques might cause the neuropathology that leads to the characteristic dementia of this disease. One compelling hypothesis that is supported by substantial experimental data postulates that senile plaques cause glial cell activation, resulting in the release of a host of glial-derived molecules that contribute to disease progression.

Immunohistochemical analyses reveal a distinct and robust glial response in the immediate vicinity of senile plaques. Notably, activated microglia (14,15) that express surface major histocompatibility antigens (16) are found associated with the plaque core. Moreover, glial fibrillary acidic protein (GFAP)-positive reactive astrocytes circumscribe senile plaques (17). The localization of activated glia is relatively specific to the area near the senile plaques, and it is particularly noteworthy that activated microglia and astrocytes are not typically located at diffuse plaques (18). These deposits contain amorphous $A\beta$ aggregates and are generally devoid of neuritic damage. This suggests that the glial cells respond specifically to $A\beta$ fibrils and subsequently promote neuritic damage. This premise is supported by the observation that there is a positive correlation between activated glia and the presence of tau-2-positive neuritic plaques (19).

Based on the above mentioned findings, as well as many further reports on amyloid and glial phenomena in AD, we and others have proposed hypotheses for the possible role of activated astrocytes and microglia in the etiology of AD (20–24). Hypotheses are of little benefit unless they can be tested and discarded or refined. Therefore, we will try to review the progress made over the last few years in testing and evaluating a number of these hypotheses. It is beyond the scope of this chapter to review the

progress of all hypotheses concerning the role of neuroglia in AD and we will thus focus on two promising concepts which have the benefit of being testable and which have received much confirmatory attention over the last half-decade. The first is the modified excitotoxicity hypothesis in which β -amyloid is proposed to induce its neurotoxicity indirectly by an inhibitory action on the uptake of synaptic glutamate by astrocytes (21). The second is the immuno-inflammatory hypothesis, which postulates that activated microglia and their various secreted products are the intermediaries of the A β -induced neurotoxicity presumed to lead to the dementia of AD (22,23). These two hypotheses are not mutually exclusive, nor do they exclude other contributions to the pathology of AD, but both provide very promising approaches to providing new means of treatment for the various pathologies of AD.

The remainder of this chapter summarizes and discusses the body of literature that addresses these possible roles of glial cells in the pathology of AD. There are compelling data to support the concept that therapeutic strategies aimed at reducing the extent of glial activity in the AD brain should be tested in controlled clinical trials.

2. EXCITOTOXICITY MEDIATED BY AMYLOID-COMPROMISED ASTROCYTES IN AD BRAIN

It was proposed almost 8 years ago that A β may induce glutamate excitotoxicity in AD brain by an indirect mechanism. This hypothesis (21) suggested that neuronal cell death in AD may be secondary to an effect of A β on astrocytes in AD brain, impairing their ability to remove glutamate from neuronal synapses. Much circumstantial evidence supported this premise, but the critical experimental test of whether or not A β could indeed impair glutamate uptake by astrocytes had not yet been demonstrated. In subsequent years, a number of groups have demonstrated that A β does indeed impair the uptake of glutamate by astrocytes in culture (25–27), thus strengthening this hypothesis. Although the mechanism of the amyloid-induced impairment of astrocytic glutamate uptake has not been definitively established, it has been suggested that it may be secondary to the action of free radicals, nitric oxide (NO) or arachidonic acid (23,25), the production of which by activated neuroglia in AD brain is discussed later in this chapter.

The observations of A β -induced attenuation of glutamate transport seem particularly pertinent when combined with the understanding that glutamate is the major excitatory neurotransmitter in brain regions such as hippocampus and frontal cortex that experience significant neuronal cell loss in AD (28,29). Moreover, it has been reported that glutamate uptake is decreased in the brains of AD patients (30).

Klegeris et al. (31), furthermore, report that A β (1–40) significantly increased the extracellular glutamate levels in co-cultures of THP-1 monocytes and U-373 MG astrocytoma cells whereas the antiinflammatory drug dexamethasone significantly decreased these levels. The authors conclude that inflammatory stimuli may increase extracellular glutamate whereas antiinflammatory drugs decrease it. This group had previously reported that another antiinflammatory agent, indomethacin, significantly reduced the progression of the symptomatology of AD (32) and a mechanism has been suggested whereby this may have been due to a reduction in glutamate excitotoxicity (23). An immuno-inflammatory contribution to the pathology of AD has gained strong support and the studies above suggest that the excitotoxin, glutamate, could be a signif-

icant contributor to the inflammatory pathology in AD. Other factors contributing to the immunoinflammatory pathology are discussed in the following sections.

3. IMMUNO-INFLAMMATORY PATHOLOGY IN AD

3.1. Glial-Derived Proinflammatory Molecules in AD

3.1.1. Cytokines

Activated microglia within the AD brain have a morphology that is similar to peripheral macrophage. The prevailing view is that microglia are cells of the monocyte/macrophage lineage that migrate to the brain during early development, prior to formation of an intact blood-brain-barrier (33). Like macrophage, microglia are capable of expressing the proinflammatory cytokines interleukin-1 α (IL-1 α), IL-1 β , IL-6, and tumor necrosis factor α (TNF α). Griffin and colleagues (34) reported a 30-fold increase in the number of IL-1-positive microglia in the AD brain relative to age-matched control tissue, suggestive of a chronic inflammatory condition. There is also evidence that the levels of IL-1 β within cerebrospinal fluid may be increased in individuals with AD (35,36). Further proof of an inflammatory component in AD is provided by the observation that IL-6 levels appear to be increased in the AD brain (35). Finally, it has been reported that serum TNF α levels are elevated in AD patients (37).

As noted in the introduction, the specific activation of microglia and astrocytes near senile plaques implies that these cells are responding to one or more plaque components. Several *in vitro* studies have revealed that A β peptide is capable of eliciting a robust upregulation of cytokine expression by microglia and macrophage. Exposing mouse microglia to A β results in increased mitosis and an associated potentiation of IL-1 release (38). More recent studies have found that either nonstimulated mouse microglia (39) or those treated with γ -interferon (40) respond to A β fibrils by increasing their release of TNF α . It appears that the response of microglia and macrophage to A β is specific to the fibrillar form of the peptide. For example, human THP-1 monocytes that are differentiated to a macrophage phenotype increase their release of IL-1 β when treated with A β fibrils (41). However, no cytokine elevation was observed upon addition of nonfibrillar A β to the THP-1 cells. Recent data reveal that these cells also increase their release of certain inflammation-associated chemokines in response to A β fibrils (42).

A localized, chronic increase of IL-1 in the AD brain is likely to lead to changes in astrocyte expression of IL-6, as evidenced by the significant release of IL-6 by these cells in culture after addition of IL-1 (43). A β fibrils further enhance IL-1-induced IL-6 release from astrocytes (44). Thus, it is possible that the reported IL-6 elevation in AD (35) is due to both microglial-derived IL-1 and plaque-associated A β fibrils.

Astrocytes also respond to IL-1 by increasing the expression of S100 β (19), a protein with growth-promoting activity that has been suggested to contribute to the formation of dystrophic neurites around senile plaques. The reactive astrocytes found in the vicinity of plaques overexpress S100 β (34), and levels of S100 β were found to be significantly correlated with the number of tau-2-positive neuritic plaques (45). Furthermore, transgenic mice that overexpress S100 β show an age-dependent increase in cytoskeletal abnormalities that may be related to those observed in AD (46). Potential neuropathological effects of S100 β may result not only from its ability to provoke neuritic growth, but also from a potentiation of astrocyte NO production (47).

IL-1 may further contribute to AD pathology by causing increased production of astrocyte-derived α 1-antichymotrypsin (ACT) (48), a protein that may enhance the assembly of A β fibrils (49). In addition, several studies have demonstrated that IL-1 can increase APP expression (50,51). The release of IL-1 by activated microglia in AD could thus initiate a vicious cycle in which additional A β peptide is formed from APP, with ACT potentially contributing to greater plaque deposition and resulting glial activation.

Finally, an elevation of proinflammatory cytokines in the AD brain could lead directly to neuropathology. The combination of TNF α and IL-1 causes neuronal death in culture (52) and transgenic mice that express elevated levels of IL-6 in their brain show severe neuron loss as they age (53). In summary, a chronic release of inflammatory cytokines by glia in the AD brain could readily contribute to the neuronal damage and death that leads to the dementia of this disease. This concept has recently been bolstered by the finding that a genetic variation in the IL-6 gene reduces the risk of AD (54).

3.1.2. Arachidonic Acid and Eicosanoids

Among the variety of potential detrimental effects triggered by IL-1 elevation in the AD brain may be the production of arachidonic acid and its metabolites. Astrocyte cultures that are treated with IL-1 increase their release of prostaglandin E2 (PGE2) (55). In fact, IL-1-induced IL-6 release by astrocytes has been suggested to be mediated by PGE2 (56). An IL-1-initiated elevation of PGE2 may result from an upregulation of phospholipase A2 (PLA2), the enzyme responsible for the release of arachidonic acid, since both IL-1 and TNF α cause increased expression of PLA2 by astrocytes (57–59).

In addition to serving as a prostaglandin precursor, arachidonic acid could contribute to AD neuropathology by additional mechanisms. This fatty acid has been implicated as a direct neurotoxin (60) and is an effective inhibitor of high-affinity glutamate uptake by astrocytes (61,62). As noted above, because astrocytes are the primary regulator of glutamate concentrations near NMDA and AMPA receptors, a reduction in their ability to remove glutamate could lead to excitotoxic neuronal damage in the cognitive regions of the brain (21,23).

Consistent with the possibility of microglial IL-1 release leading to enhanced PLA2 activity and consequent arachidonic acid release is the finding that astrocyte PLA2 immunoreactivity is increased around senile plaques in AD (63). There is also evidence of cyclooxygenase-2 elevation in neurons in AD (64), leading to the possibility of astrocyte-derived arachidonic acid being converted to eicosanoids by neurons in the AD brain.

3.2. Glial-Derived NO and Free Radicals

Glial cells may further contribute to neuropathology in AD through their release of agents that lead to free radical damage. There is evidence of oxidative damage in the vicinity of senile plaques in AD (65,66) and microglia are known to release increased levels of superoxide anion into culture medium after exposure to A β fibrils (40,67). This suggests that the microglia that are in intimate contact with senile plaques might release superoxide and other free radicals that are detrimental to neurons.

An additional glial-derived agent that can result in oxidative damage is NO, which can form the highly toxic peroxynitrite ion upon interaction with superoxide (68). It

has recently been revealed that there is an upregulation of inducible NO synthase (iNOS) in the reactive astrocytes that are adjacent to A β -containing plaques in AD (69). This elevation in astrocyte iNOS could result from the actions of cytokines and/or S100 β which, as discussed under **Subheading 3.1.1.**, are elevated in AD. In vitro studies have documented that the combination of IL-1 and TNF α result in enhanced NO production by astrocytes (70). Likewise, treatment of astrocyte cultures with S100 β results in iNOS activation and NO release (47). Because astrocyte synthesis of S100 β in AD may be triggered by microglial-derived IL-1, a compelling argument can be made that A β activation of microglia and a resulting release of IL-1 and TNF α may start a cascade of events that ultimately leads to the production of additional neurotoxic agents such as S100 β , IL-6, arachidonic acid, superoxide anion, and NO.

3.3. Glia as Potential Sources of Complement Proteins

The early activation products of the classical complement pathway (i.e., C1 and C4) are found associated with senile plaques in AD brain (71,72). Moreover, the terminal complement activation product, membrane-attack complex (MAC or C5b-9), appears to be localized to dystrophic neurites found in the vicinity of senile plaques (73,74). These observations have resulted in the hypothesis that complement activation may contribute to neuronal damage in AD.

Although the classical complement cascade may progress to completion around senile plaques, it appears that the pathway only proceeds through C3 in diffuse plaques (71). The apparent absence of later complement components, such as MAC, at diffuse plaques suggests that there may be relatively low levels of one or more of the C5–C9 precursors in the region of these deposits. The complement seen in AD brain is likely synthesized by resident central nervous system cells since there does not appear to be a general disruption of the blood-brain-barrier (75). *In situ* hybridization studies indicate that microglia and neurons of the AD brain contain elevated amounts of C1q and C4 mRNA, respectively (76). Both astrocytes and microglia can synthesize most of the classical pathway proteins in vitro (77,78). It is therefore possible that the activated glia found at senile plaques synthesize key complement proteins, and the absence of these cells in the vicinity of diffuse plaques may account for the observed shortage of later complement proteins and activation products. If this is true, then agents that suppress plaque-associated glial activation should prove useful in attenuating any damage associated with complement activation in the AD brain.

4. CONCLUDING REMARKS

The findings discussed above suggest a number of potential approaches that might be employed to minimize the contribution of glia to neuronal damage in AD. Since several reports indicate that the interaction of A β fibrils with microglia triggers cellular activation, it would seem desirable to block this amyloid-associated event. It has been suggested that A β can bind to specific receptors on microglia, including the scavenger receptor (67) and the receptor for advanced glycation end-products (RAGE) (39). Hence, molecules that prevent amyloid peptide binding to one or both of these receptors may reduce microglial activation at senile plaques and consequently be of therapeutic value. However, there is some question as to whether either of these receptor systems are truly responsible for initiating the immuno-inflammatory events described

in this chapter (79,80), and the potential effectiveness of RAGE or scavenger receptor antagonists in AD awaits further testing.

Since many of the changes in glial cell protein expression in AD may result from an initial release of IL-1 and other proinflammatory cytokines, compounds that reduce inflammation would seem to be reasonable choices as potential drug candidates. In fact, there are several epidemiological studies which conclude that classical antiinflammatory drug treatments reduce the onset of AD (reviewed in (81,82)). A confounding factor in utilizing existing steroidal or nonsteroidal antiinflammatory drugs (NSAIDs) in AD is the general intolerance of the elderly to these compounds. Thus, newer drugs without the side-effect profiles of typical antiinflammatory treatments are needed to serve the AD population. Furthermore, current NSAIDs are primarily cyclooxygenase (COX) inhibitors that affect cytokine expression only at very high concentrations (83). Although COX inhibition would protect against any detrimental effects related to arachidonic acid metabolites in the AD brain (*see Subheading 3.1.2.*), NSAIDs would be predicted to be relatively ineffective at attenuating glial cytokine release. Accordingly, specific inhibitors of proinflammatory cytokine expression may prove to be more valuable in slowing AD progression.

In addition to cytokines, activated microglia and reactive astrocytes in the AD brain appear to release a variety of additional harmful molecules. It is difficult to predict the relative contribution of these various bioactive species to disease neuropathology. As discussed, free radicals, NO, glutamate, arachidonic acid and/or complement proteins may play a role in neuronal damage in AD. Thus, drugs directed toward one or more of these downstream targets may prove to be efficacious. For example, antioxidants, glutamate receptor antagonists, PLA2 inhibitors or inhibitors of complement activity could reasonably be postulated as possible drug candidates for AD. The next several years promise to be very interesting since it is likely that one or more of these approaches will be pursued clinically in the hopes of slowing the glial contribution to AD.

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Reactive Astroglia in the Ataxic Form of Creutzfeldt-Jakob Disease

Cytology and Organization in the Cerebellar Cortex

Miguel Lafarga, Nuria T. Villagra, and Maria T. Berciano

1. INTRODUCTION

Transmissible spongiform encephalopathies (TSE) are sub-acute neurodegenerative disorders which include scrapie in sheep, bovine spongiform encephalopathy in cows, and Creutzfeldt-Jakob disease (CJD), kuru, Gerstmann-Straussler syndrome, and fatal familial insomnia in humans. The essential pathogenic component of TSE is an abnormal isoform of the prion protein designated PrP^{Sc} (for reviews and discussions *see 1*). The naturally occurring, cellular prion protein, called PrP^C, encoded by the *Prnp* gene, is expressed in neurons (2) and glia (3). PrP^C is glycosylated and anchored to the plasma membrane, but little is known about its cellular function (*see references in 1*). During the disease process, PrP^C is converted into PrP^{Sc} in both neurons and astrocytes. The “converted” PrP^{Sc} is partly resistant to proteinase K digestion and has an altered conformational state whereby the amount α -helical structure decreases and the content of β -sheet increases (for review *see 1*). Prion-induced encephalopathies are characterized by intracerebral accumulation of PrP^{Sc} and deposition of PrP amyloid (for review *see 1,4*). The observation that *Prnp* knockout mice do not develop spontaneous scrapie (5) supports the view that the accumulation of PrP^{Sc} and PrP amyloid in the brain mediate neuronal toxicity in the central nervous system.

Common neuropathological hallmarks of prion diseases are neuronal degeneration, spongiform changes, formation of amyloid plaques, and astrogliosis (for review *see refs. 6–8*). The reactive response of astrocytes includes cellular hypertrophy and hyperplasia (9,10). Although the pathogenesis of the astrogliosis in TSE is still undetermined, some factors such as the neuronal damage (9) and the effect of the tumor necrosis factor α (11) and other factors released by microglia (12) seem to be involved in the astroglial reaction. In addition, several experimental studies have demonstrated in scrapie-infected mice and hamsters that PrP^{Sc} accumulates in astrocytes prior to the development of neuronal vacuolation and reactive gliosis (13–16). Whereas the conversion of PrP^C to PrP^{Sc} in neurons leads to degeneration and neuronal death, in astrocytes it precedes upregulation of glial fibrillary acidic protein (GFAP), suggesting that hyper-

trophy and proliferation of astroglia occurs as a response to the presence of PrP^{Sc} (for review see 15,16). In fact, recent studies in vitro have shown that PrP^C-expressing astrocytes proliferate in response to a neurotoxic peptide corresponding to residues 106–126 of human PrP (PrP 106–126), a sequence which is included in the protease-resistant fraction of the PrP^{Sc} (12,17).

The current presentation will focus on the astrogliosis seen in the cerebellar cortex in three cases of the rare ataxic form of CJD with different degrees of neuronal loss. This ataxic form of CJD causes a progressive neurodegenerative disorder of the cerebellar cortex with loss of granule cells (18), diffuse isomorphic astrogliosis (19–21), upregulation of the GFAP gene expression (9) and deposition of PrP in the granular layer (22). Apart from the possible direct effect of the PrP^{Sc} on the astroglial response, the ataxic form of CJD offers a useful pathological system of granule cell depletion to investigate the plasticity of neuron-astroglia interactions in response to acquired neuronal loss in a noninvasive injury of the cerebellar cortex. An additional advantage of this region is the geometrical cytoarchitecture of both neurons and astroglia. The latter includes two astroglial subtypes, Bergmann glia and granular layer astrocytes, which are segregated in different cortical layers and interact differently with each type of neuron. Thus, Bergmann glia establishes major interactions with Purkinje cells and granule cell axons, the parallel fibers, whereas astrocytes are involved in the clustering of granule cell perikarya and in the formation of perisynaptic glia around cerebellar glomeruli (23).

2. NEURONAL DEATH AND NUMERICAL DENSITY OF NEURONS AND ASTROGLIAL CELLS IN THE CEREBELLAR CORTEX OF CJD

A neuropathological hallmark of the cerebellar cortex of the three cases studied of CJD is the severe loss of granule. Estimation of the numerical surface density of granule cells per mm² of granule cell layer has shown a range of reduction between 40% to 60% in CJD cerebella with respect to the controls (18,21). The massive depletion of granule cells results in the virtual disappearance of the characteristic clusters or rosettes of granule cell perikarya (Figs. 1 and 2). Furthermore, numerous degenerating granule cells with pyknotic nuclei and spongiform changes in the neuropil are observed (Fig. 2). These findings indicate that granule cells are particularly vulnerable to the prions in the ataxic form of CJD, which is also supported by the PrP deposition in the granular layer (22). The quantitative estimation of the astrocyte-granule cell ratio in the granular layer has registered a dramatic change from 1:98 in human controls vs 1:22 to 1:17 in the CJD cerebella (18). This increased density of astrocytes may simply be due to the higher concentration of this glial population following granule cell loss and subsequent reduction in cerebellar size. However, our observation in all cases of CJD, but not in control cerebella, that a few reactive astrocytes express vimentin (18,21), a marker of immature differentiating astrocytes (24,25), suggests that a minor proliferation of astrocytes or their precursors takes place in the CJD cerebella. In agreement with this interpretation, Biernat et al. (10) have reported that a small fraction (less than 5%) of reactive astrocytes show proliferating cell nuclear antigen (PCNA) immunoreactivity in mice with experimental CJD. Furthermore, recent studies in vitro have also demonstrated that PrP^C-expressing astrocytes proliferate in response to the prion peptide PrP 106–126 (12,17).

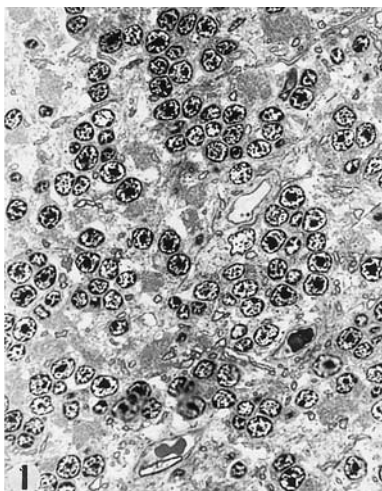


Fig. 1. Semithin section stained with toluidine blue of the granular layer from a control cerebellum showing the typical clusters of granule cell perikarya. $\times 900$.

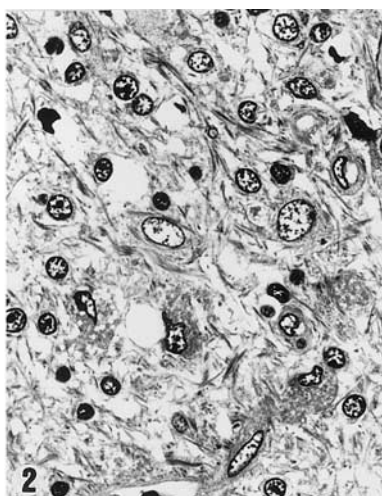


Fig. 2. Semithin section of the granular layer from a CJD cerebellum. Severe spongiosis and depletion of granule cells is seen. Numerous pyknotic nuclei of degenerating granule cells and two hypertrophied astrocytes are also observed. $\times 900$.

We have also estimated the numerical density of Purkinje cells and Bergmann glial cells per mm of the Purkinje cell layer and their ratios in two cases of CJD (CJ-1 and CJ-2) and two controls. Although in the CJ-1 the number of Purkinje and Bergmann glial cells remained unchanged with respect to controls (Purkinje cell-Bergmann glia ratio 1:10), a significant reduction (about 32%) in Purkinje cell density accompanied by an increase (about 35%) in Bergmann glia density was detected in the CJ-2 (Purkinje cell-Bergmann glia ratio 1:20). This provides the opportunity to evaluate the reac-

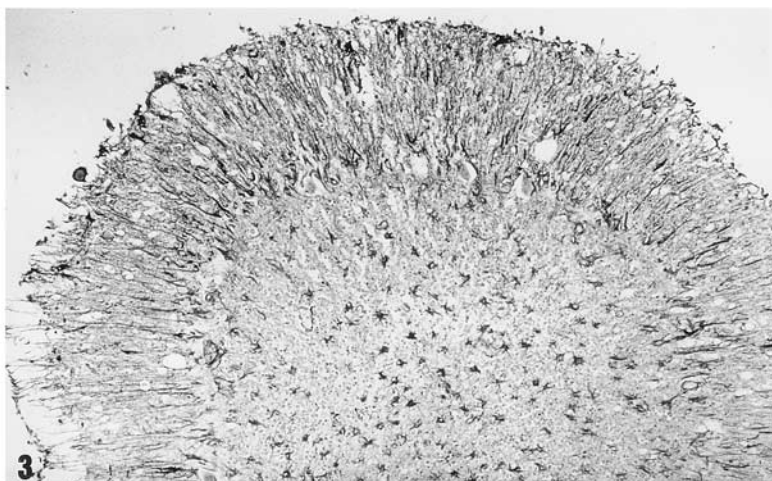


Fig. 3. GFAP immunocytochemistry of a sagittal section of the cerebellar cortex from a case of CJD. The molecular layer shows a hypertrophied palisade of normally oriented Bergmann fibers. In the granular layer the hypertrophied astrocytes appear regularly arranged. $\times 125$.

tive response of Bergmann glia to the partial loss of their specific neuronal partner, the Purkinje cells (*see Subheadings 3–6*).

3. CYTOARCHITECTURE OF REACTIVE ASTROGLIA IN THE CEREBELLAR CORTEX OF CJD

In the normal cerebellar cortex, the cytoarchitecture of Bergmann glial cells and granule cell layer astrocytes is clearly demonstrated by using GFAP immunostaining. In all cases of ataxic form of CJD studied, we have found (18,21) a marked astroglial hypertrophy and overexpression of GFAP, but the normal geometry and spatial arrangement of both astroglial subtypes in the cortical layers appears strictly preserved (Figs. 3–5). Thus, in the molecular layer Bergmann fibers show a dense palisade organization of straight and hypertrophied processes ascending throughout this layer. The regular alignment of Bergmann fibers is clearly seen in semithin, $1\mu\text{m}$ thick, sagittal and horizontal cerebellar sections immunostained with the GFAP antibody (Figs. 4A,C). However, the comparative analysis of the cases CJ-1 and CJ-2 with preservation and loss of Purkinje cells, respectively, show that the degeneration and loss of Purkinje cells in CJ-2 results in a greater cellular hypertrophy of Bergmann glia with very robust glial processes (Fig.4B). Although in this case there was an increased numerical density of Bergmann glia, vimentin immunocytochemistry does not allow us to infer a possible hyperplasia because, unlike granule cell layer astrocytes, mature Bergmann glia constitutively express this cytoskeletal protein (26). However, the normal numerical densities of both Purkinje cells and Bergmann glia found in the CJ-1 support that, at least in this pathological case, the astroglial reaction does not involve proliferation of Bergmann glia or their precursors (18) and may be partially induced by the granule cell loss and subsequent denervation of dendritic spines of Purkinje cells. In the CJD cerebella, the few Fañanas glial cells found in the upper portion of the molecular layer also exhibit a reactive appearance with prominent GFAP expression (Figs. 4D,E).

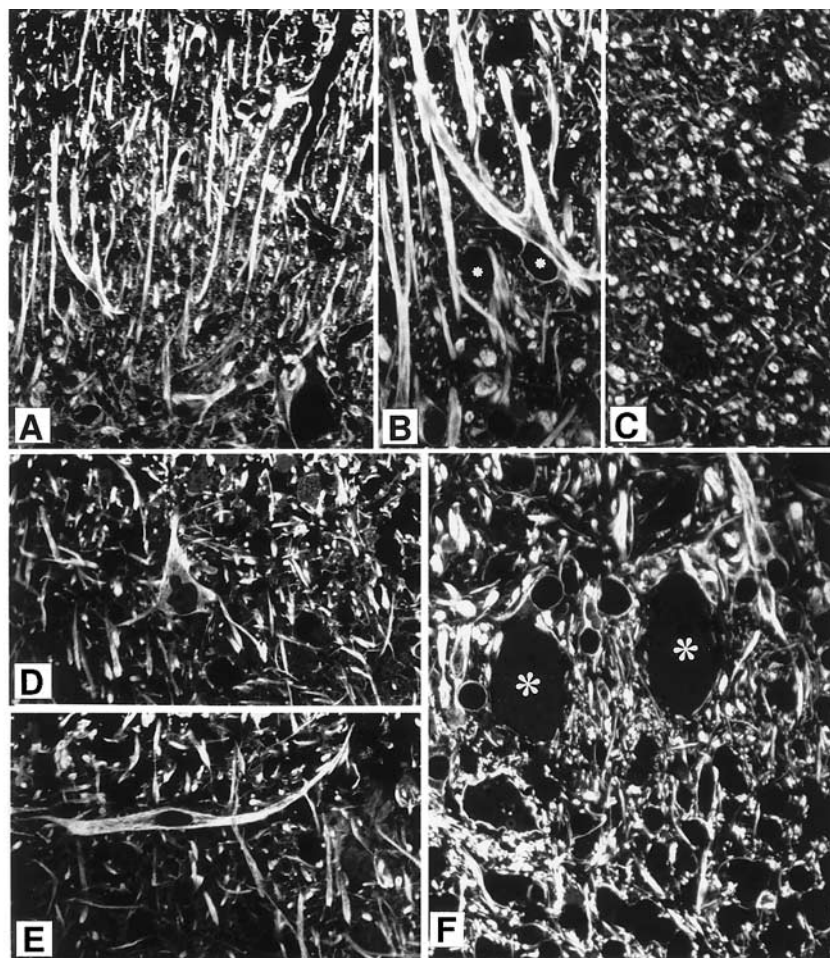


Fig. 4. GFAP immunofluorescence on 1 μ m thick semithin sections of the cerebellar cortex from CJD cerebella. (A) and (B) Sagittal sections illustrating the dense palisade of straight, very thick Bergmann fibers. Note in (B) the hypertrophied Bergmann glia perikarya (asterisks). (C) Horizontal section of the molecular layer showing the regular arrangement of cross-sectioned GFAP positive Bergmann fibers. (D) and (E) Examples of hypertrophied GFAP positive Fañanas cells in the upper half of the molecular layer. (F) Purkinje cell layer showing a dense perineuronal net of immunofluorescent glial processes surrounding Purkinje cell perikarya (asterisks). In the underlying granule cell layer brilliant astrocytic processes delineate the unlabelled granule cell perikarya. (A) \times 750, (B–F) \times 1000.

Other interesting findings in the CJD are the reinforcement of the astroglial perineuronal nets that cover Purkinje cell perikarya (Fig. 4F) and the formation of a plexus of basal processes of Bergmann glia located at the interface between Purkinje and granular layers (Fig. 5B). This latter component of the glial response to neurodegeneration tends to create a barrier between these two cortical layers that may preserve the specific functional domains of Bergmann glia and astrocytes in molecular and granular layers, respectively.

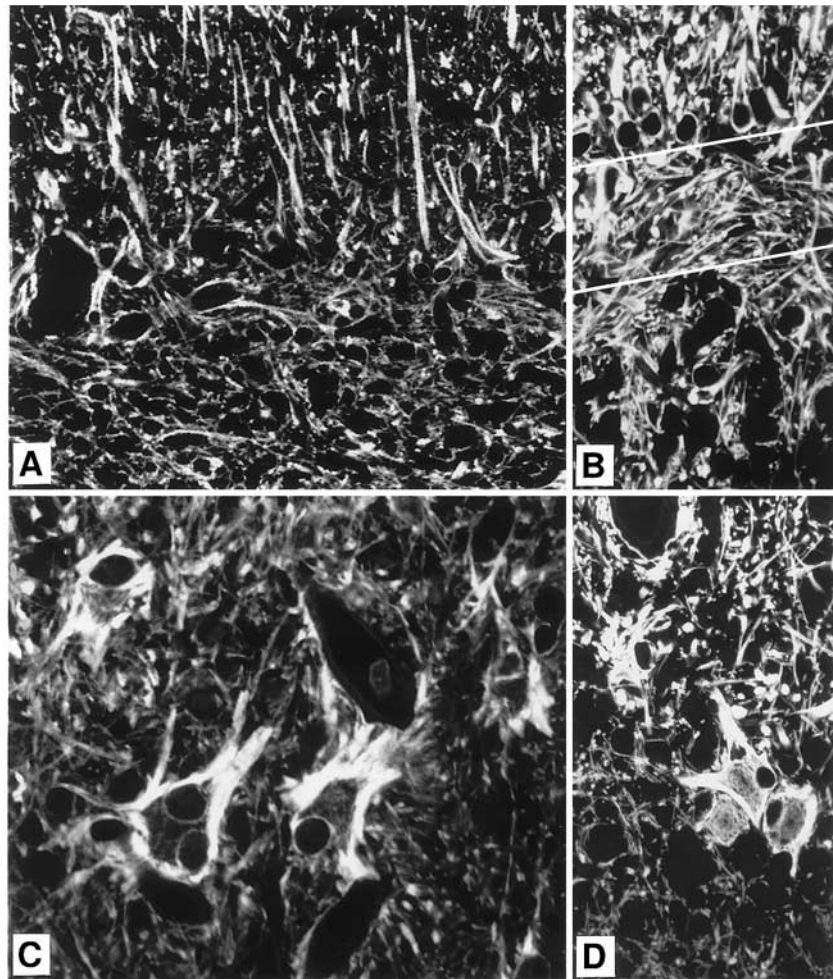


Fig. 5. Semithin sections of the cerebellar cortex immunostained with a GFAP antibody from CJD cerebella. **(A)** and **(B)** These pictures illustrate molecular-Purkinje-granule cell layers transition. The upper region of the granular layer seen in **(A)** shows a dense network of GFAP immunofluorescent astrocytic processes demarcating granule cell perikaryon compartments. **(B)** A dense basal plexus of Bergmann glia (within white lines) is observed at the transition between Purkinje cell and granule cell layers. **(C)** Detail of reactive astrocytes with perivascular endfeet in the granular layer. Note a binucleolated cell and the very intense GFAP staining of the marginal cytoplasm. **(D)** A cluster of hypertrophied astrocytes. A network of prominent profiles of reactive astrocytes is visible throughout the granule cell layer neuropil. **(A, B and D)**, $\times 750$, **(C)** $\times 1000$.

Regarding the granular layer astrocytes, GFAP immunostaining reveals a great hypertrophy of perikarya and cellular processes (Figs. 3, 5) which is clearly associated with the severe degeneration and loss of granule cells and their synaptic contacts with mossy fibers, the cerebellar glomeruli, in the CJD cerebella. In spite of the severe neuronal degeneration, reactive astrocytes appear regularly spaced (Fig. 3) and their hypertrophied radial processes form an extensive structural network of communication

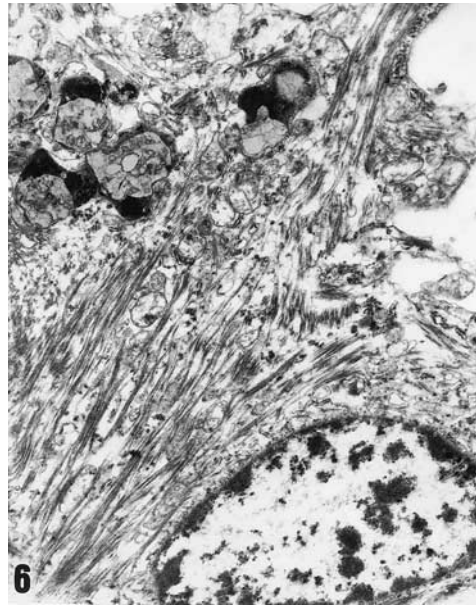


Fig. 6. Electron micrograph of the perikaryon of a reactive astrocyte with abundant bundles of glial filaments and some lipofuscin bodies. $\times 10,000$.

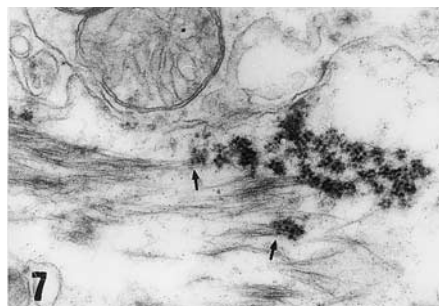


Fig. 7. Association of intermediate glial filaments with polyribosomes (arrows) in the cytoplasm of a reactive astrocyte. $\times 30,000$.

among astrocytes which is closely associated with the surviving granule cells and blood vessels (Figs. 3, 4F, 5A). This reflects that “contact spacing” (27) behavior observed among normal astrocytes is also preserved among reactive astrocytes in the isomorphic form of CJD astrogliosis. The perikaryal cytoplasm of CJD reactive astrocytes shows intense GFAP immunostaining, particularly concentrated at the marginal and perinuclear cytoplasm. Some gemistocytic astrocytes with extensive cytoplasm are also observed (Fig. 5D). Electron microscopy studies in CJD cerebella (21) have revealed the characteristic hypertrophied bundles of glial filaments crossing the perikaryal cytoplasm and running in glial processes of reactive astrocytes (Figs. 6–8). In addition, we have observed close structural relationships between glial filaments and rosettes of polyribosomes (Fig. 7), suggesting that some mRNAs are associated with

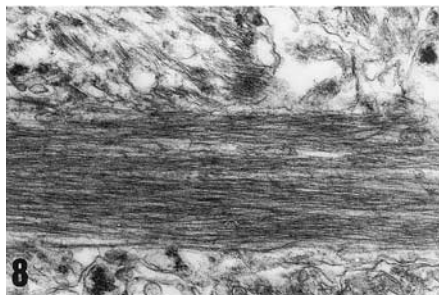


Fig. 8. Longitudinal section of a hypertrophic Bergmann fiber showing a prominent fascicle of densely packed glial filaments. $\times 30,000$.

cytoskeletal elements of reactive astrocytes, as previously described in nonneural cells (28). In this way, the intracellular localization of mRNAs is thought to play an important role in guiding protein toward appropriate cellular compartments with minimal transport costs (29).

4. NUCLEAR AND NUCLEOLAR ORGANIZATION IN REACTIVE ASTROGLIA OF CJD CEREBELLA

The reactive response of astroglia to neurodegeneration in the CJD cerebellar cortex includes a profound nuclear remodeling characterized by the increase of nuclear and nucleolar size, chromatin decondensation and formation of nuclear bodies (NBs). Karyometric analysis performed in Bergmann glia and granule cell layer astrocytes has demonstrated greater nuclear size in both cell types in the CJD cerebella with respect to normal cerebella. In addition, the level of nuclear enlargement of Bergmann glia and granule cell layer astrocytes is positively related to the degree of neuronal loss of their specific neuronal partners, Purkinje cells and granule cells, respectively (18,21). This highlights the plasticity of reactive astroglial response as a function of the neuronal loss and subsequent reduction of synaptic activity. The increase in the nuclear size of astrocytes is a normal response in all types of astrogliosis (30) associated with the cellular hypertrophy and is also observed in hypothalamic astrocytes of aged rats (31) and in reactive-like astrocytes induced in vitro by dibutyryl cyclic AMP treatment (32). Nuclear enlargement usually reflects the activation of transcriptional activity which involves increased RNA synthesis, enhanced import of karyophilic proteins and chromatin unfolding (33,34). Furthermore, the presence in the CJD of some reactive astroglial cells with very large nuclei or binucleolated (Fig. 5C) supports that cellular mechanisms of endoreduplication and endomitosis may occur resulting in an increase of ploidy.

The morphology of nuclei in reactive astroglial cells is characterized by extensive areas of dispersed chromatin (euchromatin) in the nuclear interior, the presence of small clusters of interchromatin granules, nuclear domains enriched in splicing factors for pre-mRNAs (35), and the appearance of NBs (Figs. 9–11), all parameters related to activation of transcription and RNA processing (35). NBs correspond to simple and complex nuclear bodies reported by Bouteille et al. (36) in several cell types. Although simple NBs are composed of an amorphous fibrillar material usually organized in con-

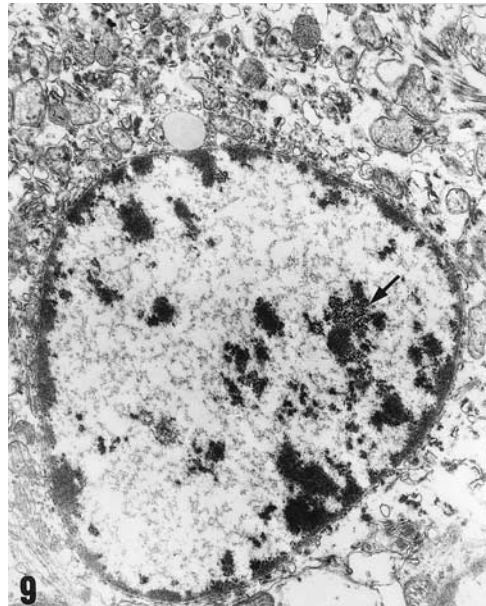


Fig. 9. A large cell nucleus of a reactive astrocyte with extensive domains of euchromatin and some small clusters of densely packed interchromatin granules (arrow). $\times 12,000$.

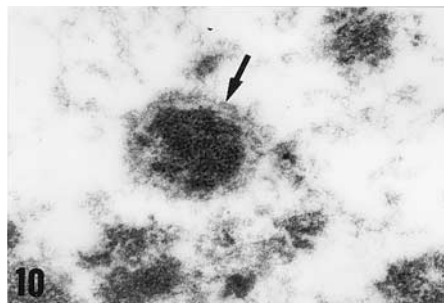


Fig. 10. Detail of a complex granular body (arrow) composed of a microfibrillar capsule and a dense granular core within the cell nucleus of a reactive astrocyte. $\times 30,000$.

centric layers or spiral configuration (Fig. 11), the complex NBs exhibit a larger size and are formed by a fibrillar capsule that encloses a dense granular core (Fig. 10). In the CJD, both types of NBs have been found in reactive astrocytes from several brain regions (21,37). They also appear in axotomy-induced reactive astrocytes (38), hypothalamic astrocytes of aged rats (31) and in reactive Schwann cells observed in peripheral neuropathies such as the Guillain-Barré syndrome (39). The formation of NBs in these glial cells seems to be related to an active metabolic state. This is consistent with several observations in nonneural cell types showing that the production of NBs or an increase in their frequency may be induced in experimental models of cellular activation, such as stimulated lymphocytes and estrogen-responsive cells (for review see 34,40). Cytochemical analysis has shown the proteinaceous nature of NBs with the additional presence of ribonucleoproteins in the granular core (34,36,39,40), but little

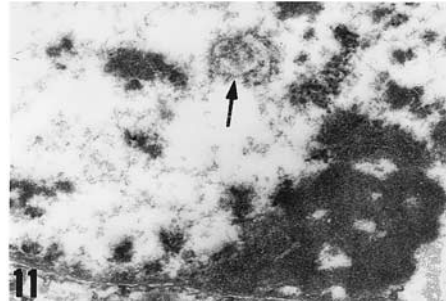


Fig. 11. Simple nuclear body formed by concentric shells of microfibrillar material (**arrow**), $\times 30,000$.

is known about their specific molecular nature. Recent immunocytochemical studies have detected the presence of a 126-kDa polypeptide, DNA polymerase α and proliferating cell nuclear antigen (41). Although the precise functional significance of NBs remains to be established, based on the content of DNA polymerase α and proliferating cell nuclear antigen in NBs, it has been proposed that they play a role in DNA replication (41). Further investigation will determine if the occurrence of NBs in some reactive astroglial cells is related to the induction of proliferation.

5. INTERASTROGLIAL JUNCTIONS AND PERIVASCULAR ORGANIZATION OF REACTIVE ASTROGLIA IN THE CJD CEREBELLA

The presence of an extensive system of interastroglial junctions, namely maculae adherens junctions, hemidesmosomes or focal contacts (plasmalemmal undercoat) and gap junctions (Figs. 12–17) is a prominent ultrastructural feature in the organization of reactive astroglia in the CJD cerebellar cortex (18,21). Maculae adherens junctions, which are rarely seen in the gray matter of normal cerebella (23), were regularly observed among cellular processes of reactive astroglia in all cases of CJD studied. They were particularly abundant among the lateral faces of perivascular endfeet (Fig. 12). Typical maculae adherens junctions appeared as buttonlike points of contact between glial processes that consist of dense cytoplasmic plaques linked to the cytosolic face of the plasma membranes of adjacent cells. Unlike hemidesmosomes, no intermediate glial filaments were anchored to the adhesion plaques. The proliferation of macular adherens junctions should increase the adhesion and structural integration among reactive astroglial cells. This may be essential to maintain the reinforced astroglial scaffold of the CJD cerebellar cortex in response to the alteration of neuron-astroglia interactions provoked by neurodegenerative changes (21). Since the normal intercellular space is preserved, these junctions do not represent a barrier to extracellular diffusion of large molecules.

The organization of perivascular glia in the CJD cerebella is characterized by the formation of hypertrophied endfeet with increased GFAP immunoreactivity (Fig. 5c) and prominent intermediate filament bundles (Figs. 15, 16). Furthermore, it has been demonstrated that perivascular endfeet are associated with thickened basal lamina and exhibit conspicuous plasmalemmal undercoat on the cell membrane facing the perivascular space (21). This undercoat, with an electron-dense and homogeneous

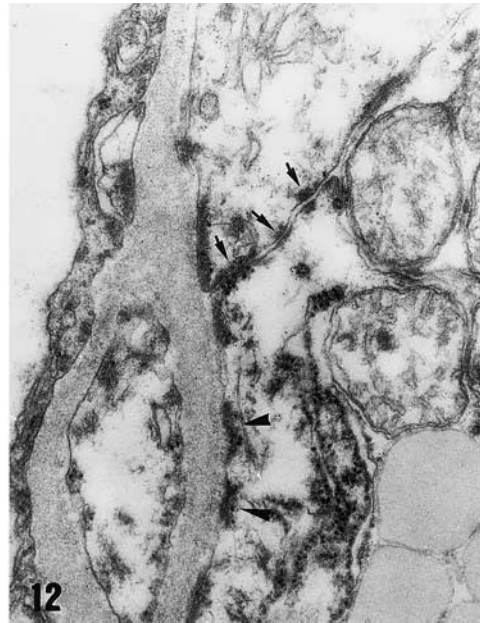


Fig. 12. Arrays of macular adherens junctions (**arrows**) and dense plaques of plasmalemmal undercoat (**arrowheads**) in perivascular endfeet of reactive astroglia. $\times 50,000$.

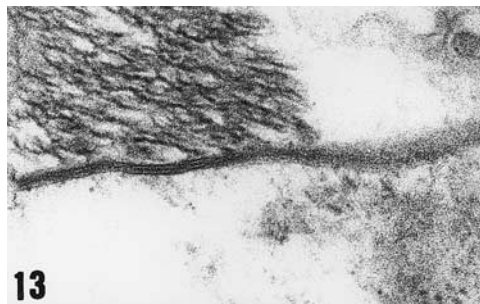


Fig. 13. A long gap junctional profile between reactive Bergmann glial processes. Note a fascicle of glial filaments in the vicinity of the junction. $\times 80,000$.

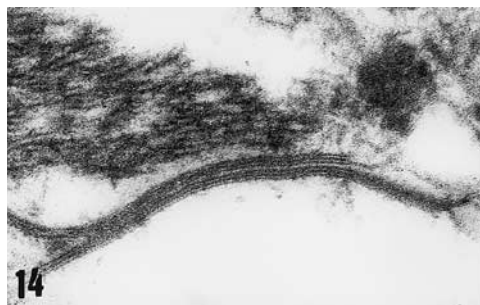


Fig. 14. Detail of paired gap junctions formed by lamellar processes of reactive astrocytes in the granule cell layer. $\times 70,000$.

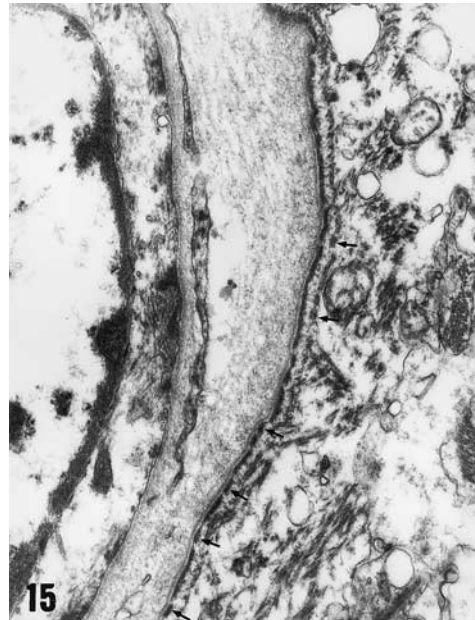


Fig. 15. Perivascular portion of the perikaryon of a reactive astrocyte around an arteriole. Note an almost continuous plasmalemmal undercoat and the terminal web of glial filaments (**arrows**) running in parallel to the plasma membrane. $\times 20,000$.

texture, is usually arranged as linear dense plaques of variable length in capillaries and venules (Figs. 12, 16), a structural configuration similar to those of dense plaques of hemidesmosomes or focal contacts, depending on the presence or absence of anchored glial filaments, respectively. In arterioles, the reinforcement of the plasmalemmal undercoat leads to dense plaques joining one another resulting in a continuous subplasmalemmal linear density (Fig. 15). In addition, perivascular endfeet frequently contain a terminal web or sheet of densely packed glial filaments which run parallel to the profile of the plasmalemmal undercoat (Figs. 15). From the terminal web, intermediate glial filaments may extend to the plasmalemmal undercoat which provides the attachment sites for this cytoskeletal component (Fig. 17). Under normal conditions, less developed subplasmalemmal densities have been observed on the pial astrocytic processes coated with the basal lamina (42). Under neuropathological conditions, well developed plasmalemmal undercoat and terminal web of glial filaments, also referred to as desmosome-like structures and anchorage densities, respectively (43), have also been reported in devastated cerebral regions with gliosis, indicating that these structural organizations are nonspecific products of reactive astroglial cells (43). Although the molecular nature of the plasmalemmal undercoat in astroglial cells is unknown, its cytological organization clearly corresponds to hemidesmosomes or focal contacts, depending on the presence or absence of anchored glial filaments, reported in nonneural cell types. In both structures integrin molecules of the plasma membrane are thought to bind to proteins within the dense plaques and to extracellular matrix protein laminin, allowing the attachment of cells to the extracellular matrix (44).

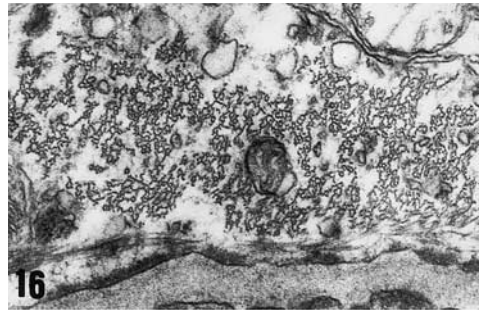


Fig. 16. Perivascular glial endfoot illustrating cross-sectioned bundles of glial filaments and several, hemidesmosome-like, dense plaques of the plasmalemmal undercoat (**arrows**). $\times 50,000$.

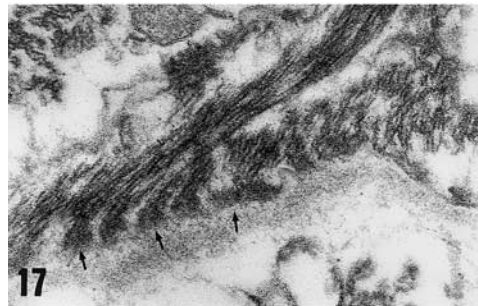


Fig. 17. Perivascular glial endfoot showing the anchorage of intermediate glial filaments to the dense plaques of the plasmalemma (**arrows**). $\times 60,000$.

In this context, the plasmalemmal undercoat with the anchored glial filaments may increase the overall rigidity and mechanical support of perivascular domains of reactive astroglia (21). Moreover, since astroglial cells induce blood-brain-barrier properties of brain endothelia (45), the reinforcement of the perivascular processes of reactive astroglia observed in CJD may contribute to protect endothelial cells against the neurotoxic effect of the PrP^{Sc}. In fact, the structure of the interendothelial tight junctions, a basic component of the blood-brain-barrier, is apparently preserved in the cerebellar cortex of CJD (21). Finally, plasmalemmal undercoat, through molecular interactions with membrane proteins, may retain astrocyte-specific complexes of integral membrane proteins, such as orthogonal assemblies of intramembranous particles (46), in the plasma membrane domains facing the perivascular space. In this way, previous freeze-fracture studies of reactive astrocytes in experimental gliosis (47), including the scrapie-inoculated mice (48), have shown a significant increase in the number of orthogonal assemblies in comparison with normal astrocytes.

Regarding interastroglial gap junctions, they are frequently found in both Bergmann glia and granule cell layer astrocytes in the cerebellar cortex of the CJD cerebella (18,21). They usually appear as long linear profiles of adjacent plasma membranes both in astroglial processes (Fig. 13) and perikarya, being particularly abundant between the lateral faces of perivascular glial endfeet. Paired gap junctions on opposite

membranes of thin lamellar astroglial processes are commonly seen in the CJD cerebella (Fig. 14). Although gap junctions are occasionally found in ultrathin sections from normal human cerebellar cortex (23), their frequent appearance in CJD cerebella suggests that the reactive state of astroglial cells induces the formation of these junctions. This is consistent with the behavior of astrocytic gap junctions in experimental models of gliosis. In the ischemia-induced brain injury, Hossain et al. (49) have shown that during the transformation to a reactive state, astrocytes undergo a gap junction remodeling with increased immunostaining for the astrocytic gap junctional protein connexin43. Similarly, reactive astrocytes involved in the formation of lesional scars are also connected by extended gap junctions in forebrain regions (50). It is now well established that gap junctions are involved in metabolic and electrical coupling between normal astroglial cells (for review see 51,52). By means of exchange ions such as K^+ and second messengers such as Ca^{2+} and IP3, astrocytic gap junctions may actively participate in the redistribution of focal extracellular K^+ increases generated by synaptic activity (51), and in the generation of intercellular calcium waves which constitute a long-range signaling system in the brain (53,54). The proliferation of gap junctions among reactive astroglial cells in the CJD cerebella suggests that there are greater requirements for intercellular signaling and metabolic cooperation between these hypertrophic glial cells.

6. PERISYNAPTIC ORGANIZATION OF REACTIVE ASTROCYTES IN THE CJD CEREBELLA

As indicated previously, the pathological hallmark of the cerebellar cortex in the ataxic form of CJD is characterized by spongiform changes, degeneration of afferent mossy fibers, severe loss of granule cells, variable degeneration of Purkinje cells, and extensive astrogliosis (19,20,55). Degeneration of mossy fibers and loss of granule cell produces a virtual disappearance of the typical rosettes of granule cell perikarya (Figs. 1, 2) accompanied by a dramatic reduction of synaptic glomeruli in the granule cell layer. In the molecular layer, a consequence of granule cell loss is the degeneration of parallel fibers resulting in a depletion of the synaptic contacts between these fibers and Purkinje cell spines (55). Remaining synapses in the CJD cerebellar cortex appear intimately associated with perisynaptic processes of reactive astroglia, which usually have a watery cytoplasm (Fig. 18). It is noteworthy in the molecular layer the existence of a numerical mismatching between presynaptic parallel fibers and postsynaptic Purkinje cell spines resulting in the frequent observation of vacant dendritic spines with preserved postsynaptic membrane densities (Fig. 19). They appear enclosed by simple or multilayered processes of reactive Bergmann glia (18). Similar vacant dendritic spines of Purkinje cells have been reported in natural occurring granule cell ectopias (56) and in neurological mutant mice with granule cell death (57), where they persist for long periods of time in the absence of synaptic transmission. This raises the speculation that the reactive Bergmann glia might maintain and signal postsynaptic sites potentially available for reinnervation (18).

Changes in synaptic activity can mediate other cellular events of the response of cerebellar astroglia to neurodegeneration in the CJD. Thus, our comparative analysis of the three cases of CJD studied has shown that the different degrees of granule cell loss and Purkinje cell degeneration, which indirectly reflect the availability of synapses in

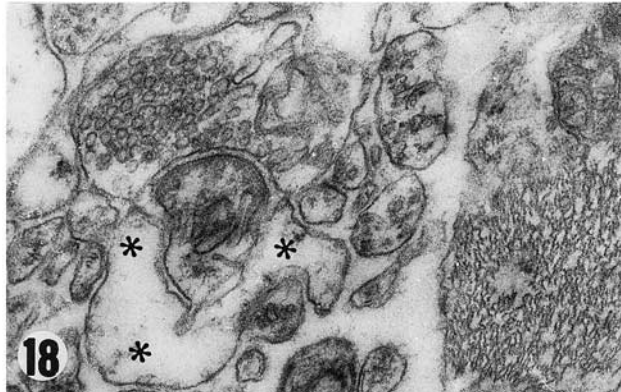


Fig. 18. A relatively well preserved synapse between a granule cell axon and dendritic spine of a Purlinje cell. Note the perisynaptic processes of Bergmann fibers (**asterisks**) and a prominent bundle of densely packed glial filaments. $\times 35,000$.

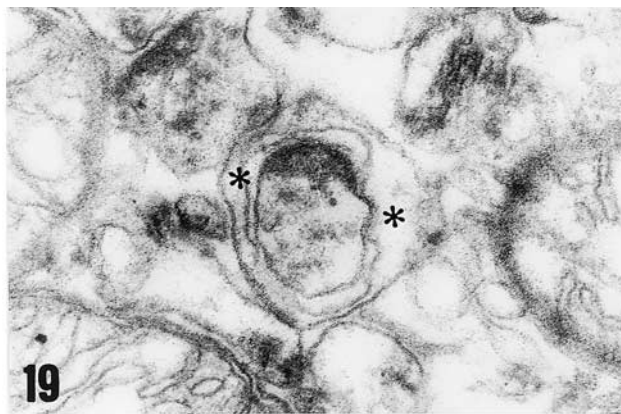


Fig. 19. A vacant dendritic spine with a prominent membrane density is surrounded by pale processes of Bergmann glia (**asterisks**). $\times 40,000$.

the molecular layer, are closely related to the levels of hypertrophy and expression of GFAP in Bergmann glia (18). In this way, a glial gene regulation by synaptic activity is an emerging concept of the last few years (for review *see* 52). Canady and Rubel (58) have demonstrated that the blockade with tetrodotoxin of afferent neural activity in chick cochlear nucleus produces a reversible astrocytic reaction with increased expression of GFAP. This effect is extensible to the model of nerve terminal and perisynaptic Schwann cell that covers the neuromuscular junction in which the deprivation of nerve activity rapidly upregulates GFAP expression in Schwann cells (59). These findings suggest that the disruption of neuron-astroglia interactions or signaling at the synapses plays a striking role in the induction of the reactive astroglial response in CJD cerebella.

In conclusion, the pattern of astrogliosis in the CJD cerebella is characterized by a reinforced astroglial scaffold which may preserve the normal geometry, spatial arrangement, and specific astroglial associations with surviving neurons. Recent data

in experimental scrapie and CJD supports that PrP may directly induce a reactive response in astrocytes (for review *see 15,16*). However, in the CJD cerebella studied here the level of the reactive astroglial response seems to be proportional to the degree of neurodegeneration and subsequent alteration of normal neuron-astroglia interactions.

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Ischemic Injury, Astrocytes, and the Aging Brain

Robert Fern

1. INTRODUCTION

Astrocytes are the housekeeping cells of the central nervous system (CNS). Their primary function is to control the environment within which neurons operate. Stroke events are characterized by a breakdown in the normal environment of neurons, leading to loss of brain function and permanent injury. Astrocytes play a critical role in this process. They are involved in the unfolding events that initiate the cellular response to ischemia, they have a significant impact upon the interactions that determine the fate of individual neurons during ischemia, and they are of central importance in the events that follow a stroke which may result in the formation of glial scar tissue. In addition to the role astrocytes play in neuronal survival during stroke, astrocytes are themselves subject to ischemic injury via mechanisms that differ significantly from those seen in neurons or other types of glia.

Stroke injury is most common in the older CNS, and the role that astrocytes play in ischemia is of greatest importance in the aged brain. The majority of basic ischemia research, however, has been performed on cultured cells taken from juvenile or neonatal animals or upon isolated tissue harvested from young animals. Bearing this in mind, I will review what is known at the basic science level about the involvement of astrocytes in ischemic injury, paying particular attention to studies that extend to the aged CNS.

Astrocytes are involved in stroke at three levels:

1. During the initial insult, where astrocytes may be damaged or killed by ischemia;
2. In the injury process itself, where astrocytes affect the fate of other cell types both in the stroke core and in the penumbra region surrounding the core and;
3. In the postischemic period where the formation of a glial scar may affect cellular reorganization and restoration of function.

Gliosis is covered in detail in a previous chapter (*see Part I: Cellular and Molecular Changes of Aged and Reactive Astrocytes*) to which the reader is directed; only reactive gliosis following ischemia will be covered here.

2. ASTROCYTES ARE SUBJECT TO INJURY DURING ISCHEMIA

Following the onset of ischemia, interruption of glucose and oxygen supply places astrocytes in a metabolically precarious position. Cellular energy reserves become

depleted and cytoplasmic ATP levels fall (1). The cell loses the ability to maintain normal ion concentration gradients across the cell membrane as the Na⁺-K⁺ ATPase fails, resulting in membrane depolarization. This series of events, which will occur in all nucleated cells during ischemia, may proceed rather slowly in astrocytes due to the presence of significant stores of the glycolytic energy reserve glycogen (2). For example, intracellular [Na⁺] increases rather slowly in cultured astrocytes exposed to simulated ischemia, rising to a concentration about 3 mM higher than resting after 60 min (3). Other important cellular events overlie the gradual degradation of cell viability resulting from ion gradient breakdown due to energy depletion. For example, in cultured astrocytes complete metabolic arrest produces a membrane depolarization over a 45 min time course that is dependent upon the presence of extracellular Ca²⁺ and is associated with a large increase in membrane input resistance (4). These changes in membrane properties are responsible for ischemic depolarization in these cells rather than a gross loss of ion gradients due to Na⁺-K⁺ ATPase failure (4). The situation in vivo is further complicated by changes in the extracellular environment during ischemia. For example, elevated [K⁺]_o will depolarize astrocytes far more rapidly than either of the two mechanisms mentioned above (5), possibly evoking cellular damage in vivo that is not seen in cultured cells (see below).

The metabolic rate of astrocytes in culture is not significantly different from that of neurons (6,7) yet ischemic conditions produce injury in cultured neurons far more rapidly than they do in cultured astrocytes. For example, Goldberg and Choi (8) have shown that withdrawal of glucose and oxygen for 70 min produces near-complete neuronal death (assessed 20–28 hr later), while 4–12 hr of glucose and oxygen withdrawal are required to produce significant death of astrocytes. The high sensitivity of neurons to ischemic conditions in vitro is due to the presence of NMDA-type glutamate receptors on the cells. These receptors are gated by an accumulation of glutamate in the bath solution during ischemia, resulting in Ca²⁺ influx and subsequent cellular injury (8). Unlike neurons (8), and oligodendrocytes (9,10) glutamate receptors do not seem to be important in astrocyte injury either in vitro or in vivo (7,11–13)

Cell culture studies such as those described above have left the impression that astrocytes are extremely resistant to ischemic injury. In vivo studies do not support this. Dead astrocytes appear in vivo after a period of ischemia as short as 30 min (14), and almost all astrocytes within the central ischemic region are dead after 4hr (14,15). At this time point the region of infarction is bordered by an area where astrocytes are actively losing their membrane integrity (14,15). This high sensitivity of astrocytes to ischemic injury in vivo compared to in vitro is a matter of considerable importance. As has recently been pointed out (16), a region of postischemic brain infarction is pan-necrotic, with all cell types dead (17). In areas where astrocytes survive ischemia but neurons die, a region of selective neuronal loss exists (18). Some restoration of function may be possible in this latter case but functional recovery will not be possible in areas of infarction. The extent of astrocyte death therefore determines the size of a region of infarction and affects the extent of unrecoverable brain function.

When the effects of ischemia are examined in astrocytes *in situ*, rapid ischemic [Ca²⁺]_i rises are observed (12,13) (Fig 1). In CNS white matter, ischemic Ca²⁺ increases are associated with cell death, with 47% of astrocytes dying during an 80 min period of ischemia (13). The cellular injury occurs as uncontrolled amounts of Ca²⁺

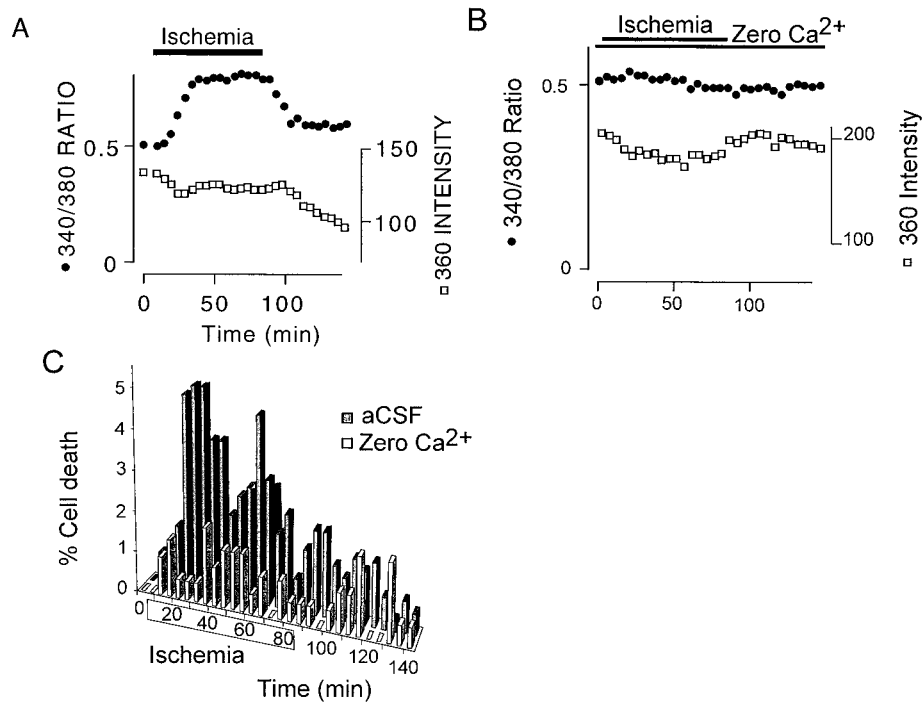


Fig. 1. Changes in $[Ca^{2+}]_i$ and cell death in astrocytes *in situ* during ischemia. Neonatal white matter astrocytes in rat optic nerve were loaded with the calcium indicator FURA-2 (see 13). (A) Following the withdrawal of oxygen and glucose ("ischemia"), the 340/380 ratio of the dye increases (filled circles), corresponding to an increase in $[Ca^{2+}]_i$. The 360 intensity of the dye (the calcium independent signal) drifts slowly lower, indicating continued cell viability in this cell. (B) In the absence of extracellular Ca^{2+} there is no increase in $[Ca^{2+}]_i$ during ischemia. (C) The occurrence of cell death (assessed as lose of 360 signal) in normal aCSF and in zero Ca^{2+} aCSF during and after an 80 min period of ischemia; demonstrating the rapid onset of cell death during ischemia, and the Ca^{2+} -dependence of the phenomena. Adapted from ref. 13 with permission.

enter the cell through voltage-gated calcium channels (VGCCs). Duffy and MacVicar (12) found that astrocytes in hippocampal slices respond to ischemia with a large rapid depolarization (about a 50 mV change after 10 min of ischemia; Fig 2). A corresponding rapid depolarization is not observed under similar conditions in cultured astrocytes (4,12), and is similar to the depolarization produced by elevating $[K^+]_o$ to 50 mM (12; Fig 2). It would appear that astrocytes *in vivo* respond to the early ischemic rise in $[K^+]_o$ with a large influx of Ca^{2+} mediated by membrane depolarization and activation of VGCCs (12,13). This phenomena may account for the much higher sensitivity of astrocytes *in vivo* (14,15) to ischemia than astrocytes *in vitro* (8). The mechanism of ischemic injury of astrocytes *in vivo* is shown in figure 3.

The mechanisms linking an ischemic rise in $[Ca^{2+}]_i$ with subsequent astrocyte death are incompletely understood, and may vary between brain regions and the time course and extent of the ischemic event. It is clear that Ca^{2+} influx is the trigger that leads to cell death since removing Ca^{2+} from the extracellular space is protective (13; Fig 1). High $[Ca^{2+}]_i$ will activate a number of destructive calcium-dependent lipases, calpains, and proteases that are harmful to cell viability (19). Generation of free radicals will also

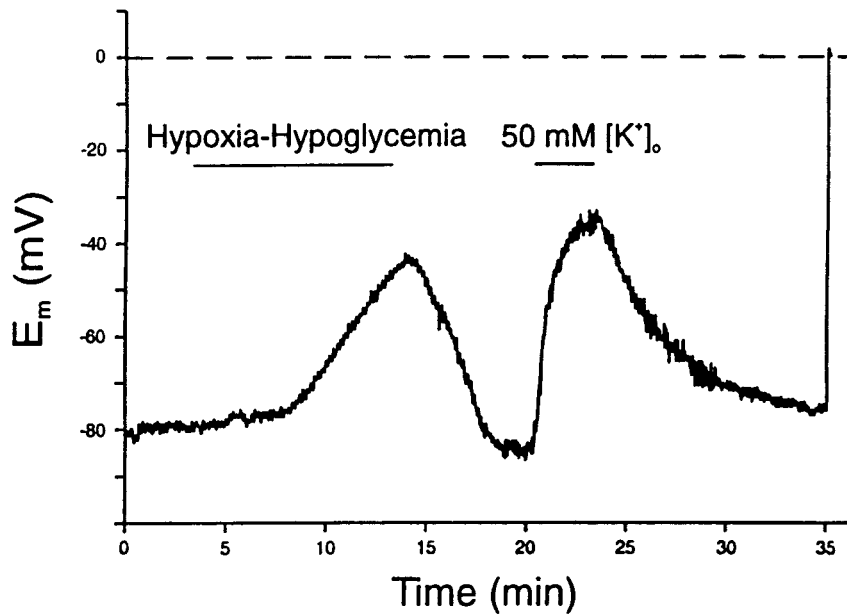


Fig. 2. Withdrawal of oxygen and glucose (hypoxia-hypoglycemia) evokes a rapid depolarization in hippocampal astrocytes *in vivo*. The degree of depolarization is similar to that produced by perfusion with 50 mM K^+ . Adapted from Duffy and ref. 12 with permission.

occur and may contribute to cell injury (20). Following the reinitiation of blood supply to an ischemic region, reperfusion phenomena occur in astrocytes that may also have important consequences for their survival. For example, removal of extracellular Ca^{2+} , as will occur during ischemia due to Ca^{2+} influx into the intracellular space, followed by return to normal extracellular Ca^{2+} gives rise to a significant Ca^{2+} influx into astrocytes (21). This phenomena (a Ca^{2+} paradox) is selectively harmful to cultured astrocytes compared to other cell types (21). A similar postischemic increase in intracellular Na^+ has been shown in astrocytes, of uncertain significance (3). Under conditions that do not lead directly to necrotic astrocyte death, high $[Ca^{2+}]_i$ is linked to the onset of apoptotic cell death (22). Apoptotic astrocytes are observed in the ischemic core and in the border zone between the core and the penumbra in ischemic lesions in mice (22); and in man (23). Apoptosis may be an important mechanism responsible for cell death in astrocytes that survive the initial wave of necrotic death.

3. ASTROCYTES IN THE INJURY PROCESS

3.1. The Initial Increase in $[K^+]_o$

Astrocytes are responsible for homeostatic control of the CNS extracellular space. Astrocytes maintain $[K^+]_o$ at around 3 mM under resting conditions, and prevent $[K^+]_o$ from exceeding about 12 mM even during the most intense periods of neuronal activity (5). During the initial stages of ischemia $[K^+]_o$ rapidly elevates above this plateau level, reaching concentrations as high as 80 mM (5). Loss of astrocyte regulation of $[K^+]_o$, and loss of the homeostatic function of these cells in general, is a key element in the onset of a stroke event. $[K^+]_o$ is likely to increase in astrocytes during ischemia (24),

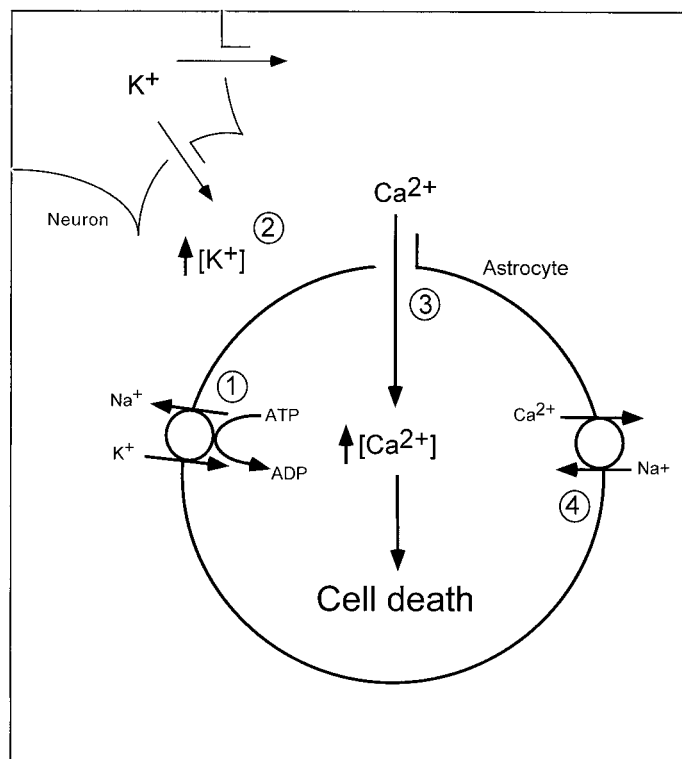


Fig. 3. A model of ischemic injury of astrocytes in vivo. During ischemia, the $\text{Na}^+\text{-K}^+$ -ATPase fails as the cytoplasmic ATP level falls (“1”). There is a rapid increase in extracellular K^+ , largely as a result of efflux from neurons (“2”). Astrocyte membrane potential is depolarized as a result, leading to the opening of voltage-gated Ca^{2+} channels (“3”). The increase in $[\text{Ca}^{2+}]_i$ that results is lethal to cell viability. Astrocytes retain the ability to remove Ca^{2+} via the $\text{Na}^+/\text{Ca}^{2+}$ - exchanger during ischemia (“4”). Adapted from ref. 13. with permission.

and the accumulation of K^+ in the extracellular space is largely a product of K^+ efflux from neurons (see Fig. 3). The capacity of astrocytes to clear extracellular K^+ is limited during ischemia due to the compromised state of the cells. For example, ischemia reduces gap-junction coupling between astrocytes (25), which will limit the ability of astrocytes to remove extracellular K^+ via spatial buffering (26). The high rate of K^+ efflux into the extracellular space during ischemia rapidly swamps any remaining ability the astrocytes have to remove K^+ , leading to high $[\text{K}^+]_o$, membrane depolarization, and release of neuroactive substances such as glutamate from neurons and glia (see **Subheading 3.4**).

3.2. Astrocyte Swelling

Astrocyte swelling is a prominent feature of CNS ischemia (24); it is the first morphological change observed (18). The mechanisms coupling these two events are complex and the situation in vivo is unclear. Movement of K^+ , Cl^- , and $\text{H}^+/\text{HCO}_3^-$ across the cell membrane, and the effects of high extracellular glutamate have all been implicated (24). Both the cell bodies and the processes of astrocytes swell during ischemia (18,27), leading to shrinkage of the extracellular space. Astrocyte swelling is associ-

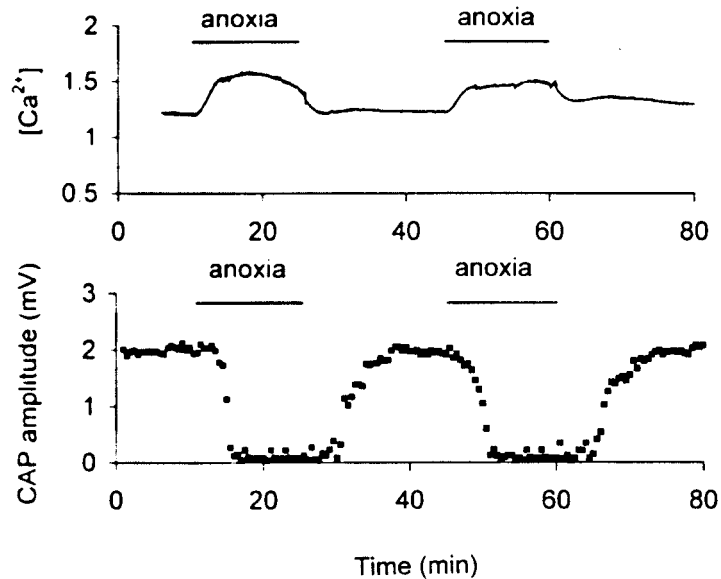


Fig. 4. Changes in extracellular $[Ca^{2+}]$ and compound action potential amplitude recorded from rat optic nerve (a CNS white matter tract). Extracellular $[Ca^{2+}]$ was measured with a Ca^{2+} – sensitive electrode, which shows a significant increase in extracellular $[Ca^{2+}]$ during anoxia. The increase in extracellular $[Ca^{2+}]$ is associated with shrinkage of extracellular space (89). Such changes in extracellular $[Ca^{2+}]$ are not as apparent in CNS gray matter during anoxia/ischemia due to the rapid influx of Ca^{2+} into cells, a process that occurs far slower in the optic nerve. Adapted from ref. 29 with permission.

ated with intracellular acidosis (28), and activation of ion channels which results in the net release of ions (24). Cell swelling is potentially protective of the astrocytes, since an increase in water content will dilute the intracellular concentration of harmful Ca^{2+} . However, extracellular space shrinkage with concomitant concentration of extracellular ions is probably detrimental to neurons during ischemia. For example, in CNS white matter $[Ca^{2+}]_o$ can increase via this mechanism during relatively brief periods of anoxia (29; Fig 4), and extracellular space shrinkage will contribute to ischemic rises in $[K^+]_o$ throughout the CNS. Since neuronal and oligodendrocyte swelling is limited during ischemia (24), such effects are primarily accountable to astrocytes. Astrocyte swelling is also associated with release of amino acids (including glutamate) into the extracellular space (30,31), which have important consequences for neurons during ischemia (see **Subheading 3.1**).

3.3. pH_o Changes

Another major dysfunction of brain homeostasis during ischemia is a prominent extracellular acidosis associated with a build up of extracellular lactate (5,32). Following the withdrawal of glucose and oxygen during ischemia, any glucose remaining in the cytoplasm together with the glycogen present in astrocytes is metabolized via glycolysis to lactate, yielding ATP (7,33,34). The lactate produced will dissociate with a pK_a of 3.84, generating a significant increase in hydrogen ion concentration. As a result, extracellular pH reaches values between 6.2 to 6.9 during ischemia (16,32,35).

In addition to lactate production in the ischemic core, the capacity of astrocytes to utilize glycolytic respiration under conditions of low oxygen in the presence of glucose (36,37), will make these cells a major contributor to lactate production in the ischemic penumbra of a stroke where such conditions exist (7,16). In addition to lactate production, other sources of ischemic H^+ production include carbonic acid and the breakdown of ATP without corresponding ATP synthesis (38).

The precise consequences of ischemic acidosis are complex, and will relate to the extent and duration of the pH change. For example, H^+ accumulation will block NMDA-type glutamate receptors on neurons, limiting the conductance of this important pathway for ischemic Ca^{2+} influx (39,40). As a result, acid pH is protective against hypoxia or glutamate exposure in cultured neurons (41,42). The presence of lactate in the extracellular space may also be beneficial to neurons under conditions of low glucose in the presence of oxygen, since it will act as an energy substrate (43,44). However, acidosis can also be directly harmful to neurons (45,46), and accentuates injury during hypoxia in rat brain slices (47,48). Hyperglycemia can either exacerbate (49,50), or ameliorate (51,52), ischemic brain injury apparently due to enhanced lactate release and acidosis; confirming the complexity of the effects of acidosis during brain ischemia (7).

Cultured astrocytes are more sensitive to the toxic effects of acidosis than are neurons (32,46). The importance of this finding is unclear, since acid levels at the extreme of those seen during ischemia are required before any significant astrocyte death is produced even if the acidosis is applied for long periods of time (16,46). The situation in vivo is also more complex than that in vitro. Swanson et al. (16) have shown significant synergy between the effects of hypoxia and acidosis upon cultured astrocyte survival (Fig 5). For example, acid pH (6.2) combined with hypoxia produce significant cell death after 5 h of exposure while under normoxic conditions this level of acidosis had no significant effect after 7 h exposure. The mechanism of this synergy may involve acid inhibition of glycolysis, upon which the cells depend during hypoxia (16). When acidosis was co-applied with high $[K^+]_o$ however, the effects of hypoxia were reduced. The interaction of environmental factors with acidosis during ischemia is not fully understood, and the importance of acidosis to astrocyte survival in vivo remains obscure. A further complication involves the potential accumulation of H^+ equivalents in astrocytes during ischemia. Kraig and colleagues (32) have provided evidence for a very low pH in astrocytes during ischemia. The consequences of this putative phenomena are not at all clear.

3.4. Extracellular Glutamate

High extracellular glutamate is a feature of brain ischemia (53), that has significant consequences for neurons (see above). As the major excitatory neurotransmitter of the CNS, glutamate is continually released from synaptice endings. Although some glutamate is taken back up by neurons, astrocytes play the major role in extracellular glutamate homeostasis (54). To fulfill this function, astrocytes express distinct glutamate uptake proteins in their cell membranes, including GLAST and GLT-1 (55). These proteins accrue intracellular glutamate with the co-transport of Na^+ and the counter-transport of K^+ and a net HCO_3^- (56,57). Membrane depolarization, high $[K^+]_o$ and high $[Na^+]_i$ during ischemia act to reduce the capacity of astrocytes to take up extracellular

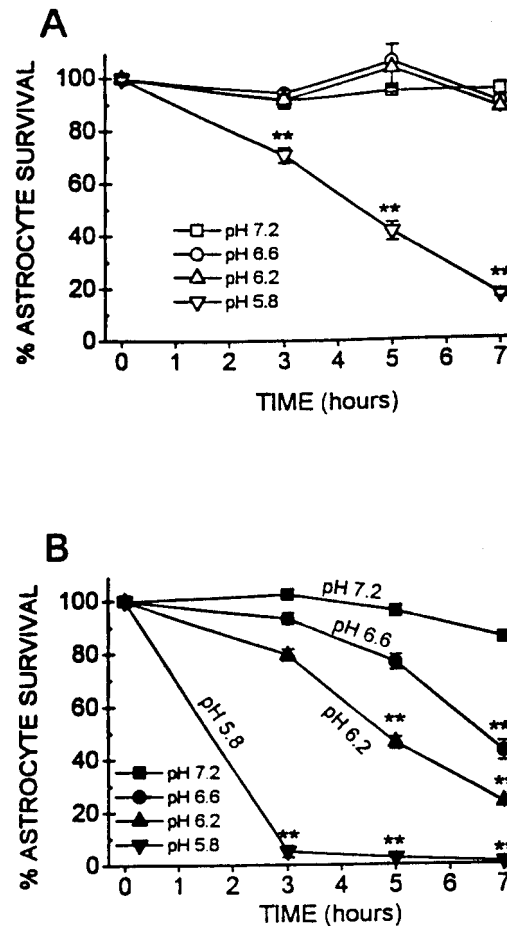


Fig. 5. Cultured astrocyte survival during acidosis is affected by hypoxia. **(A)** Astrocyte survival (assessed by lactate dehydrogenase release) over a 7-h period is affected only by very acid extracellular pH. **(B)** During hypoxia, cell death occurs at lower extracellular pH, within the range observed during ischemia in vivo. Adapted from ref. 16 with permission.

glutamate; and in fact these conditions may favor the net extrusion of glutamate from the cell since the uptake proteins are reversible (58–60). In cultured astrocytes, ischemic-like conditions reduce the capacity for glutamate uptake (11,16) and cause glutamate release (59,61). Glutamate release from cultured astrocytes is greater during ischemic conditions than that from cultured neurons (61), and there is good evidence that ischemic increases in extracellular glutamate arise either partly or largely from nonvesicular release from astrocytes (59,62).

In addition to glutamate, astrocytes play an important role in the uptake of other neurotransmitters. Many of the uptake proteins that perform this task are membrane potential and/or Na^+ dependent and have the potential to work in reverse during ischemia. Ischemia is accompanied by the extracellular accumulation of many neurotransmitters, such as GABA and glycine, that are normally regulated by astrocytes (31,62). The role these neurotransmitters play during ischemia is currently unclear, and is likely to be both important and complex (63,64). It has been argued that the ratio of

excitatory neurotransmitter release to inhibitory neurotransmitter release during ischemia might be an important factor determining the toxicity of excitatory neurotransmitter build up. Furthermore, since binding of glycine to the NMDA-receptor enhances glutamate activation of the receptor (65), glycine release may be an important determinant of the level of NMDA-receptor activation during ischemia. The role of astrocytes in the ischemic release of neurotransmitters other than glutamate may therefore be of some significance.

4. POSTISCHEMIC GLIOSIS

Following a 30 min period of ischemia induced by vessel occlusion, changes occur in astrocytes within 2 d both in regions of neuronal loss and in regions where neuronal loss is not significant (66). These changes include an increase in GFAP content and an increase in cell size. These changes only persist in brain regions that suffer neuronal loss, where they are associated with an increase in astrocyte numbers (66). In areas that do not suffer neuronal loss, reactive changes in astrocytes return to baseline. These three phenomena (increased GFAP expression, hypertrophy, and hyperplasia) are the cardinal features of reactive gliosis (32,67), with the second form described by Petito et al (66) (absence of hyperplasia) forming a separate category (32). The two types of reactive astrocytes described by Pitito et al (66) have been termed “anisomorphic” (in the immediate site of the lesion) and “somorphic” (distal from the lesion site). These terms refer to the distortion of CNS morphology produced by the generation of a glial scar at the lesion site (67). The trigger for the reactive changes seen in astrocytes following ischemia are unclear, but high $[K^+]_o$ and high pH_i are features of CNS ischemia that induce reactive changes in astrocytes (68).

The presence of a dense network of reactive astrocytes in regions of selective neuronal loss following ischemia is widely believed to be deleterious to functional recovery (69). It has been argued that reactive astrocytes form a physical barrier to the regrowth of neuronal elements such as axons (69,70). However, under some conditions, axon regrowth can be supported by reactive astrocytes (71–74) and it is possible that other factors present in the glial scar, such as oligodendrocyte or microglial cells, may be the major barrier to regrowth (67,70).

The presence of an astrocyte barrier around a lesion may act to isolate the damaged region and protect the surrounding tissue (67). Postischemic reactive astrocytes increase expression of a number of potentially protective proteins. These include metallothionein that may act as a free radical scavenger and metal ion chelator (75); glutathione peroxidase which may also protect against free radical attack (76); and ecto-5'-nucleotidase which may be protective since it increases production of extracellular adenosine (77). Other upregulated proteins may be harmful. For example, nitric oxide generation has been implicated in cell damage after ischemia (78), and a portion of the reactive astrocytes that form the postischemia glial scar are immunopositive for inducible nitric oxide synthase (79). In addition, postischemic astrocytes have elevated levels of PLA_2 , which has been implicated in delayed neuronal injury (88).

4.1. The Aging Brain

The aged brain appears to be more easily damaged during stroke (81). However, it is unclear if this is due to cellular changes at the level of neurons and glia, or whether

other age-related factors are responsible. The aged human brain exhibits slow and less profound reactive changes in astrocytes following ischemia compared to younger brain ((82). This corresponds to a lower sensitivity to oxidative stress in a model of the senescent astrocyte compared to normal cells (83). In the model used by Manganaro et al (83), cysteamine is applied to cultured rat astrocytes resulting in cellular changes similar to those seen in a population of astrocytes in the aged CNS; including increased GFAP levels and the induction of cellular inclusions. The treated astrocytes resembled closely the gomori-positive astrocytes that increase in number in the senescent brain (84).

Despite the greater predilection to stroke injury, the aged brain has a lower innate sensitivity to excitotoxic injury, apparent when an excitotoxic agent is injected directly into the brain (85,86). It is consistent with this observation that the aged brain can endure relatively high extracellular glutamate for extended periods without concomitant cell injury (87). The greater resistance at the cellular level to excitotoxic damage and the apparent higher resistance of aged astrocytes to ischemic injury correlate to a slowing of brain metabolic rate as it ages (88). A lower metabolic rate would in itself predispose the brain to a lower sensitivity to injury, although it is unclear whether this is an important factor or not. The consequences of the changes that occur in astrocytes as they age to the response of the brain to ischemia have clearly not been the subject of sufficient experimentation to date. The review of the literature presented above illustrates the important role of astrocytes in ischemic injury. Hopefully, future studies will concentrate on the role these cells play during stroke in the aging brain.

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Glial-Neuronal Interactions during Oxidative Stress

Implications for Parkinson's Disease

Catherine Mytilineou

1. PARKINSON'S DISEASE

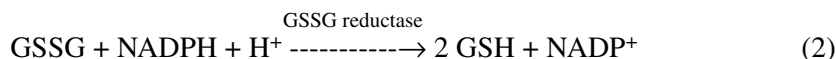
Idiopathic Parkinson's disease (PD) is a common neurological disorder of adult onset (estimated prevalence 1:1000 of the population), with tremor, rigidity, bradykinesia, and postural instability as the primary symptoms. The etiology of PD is not known. The majority of the cases are sporadic although inherited parkinsonism is reported in several families. Recently, it was discovered that a mutation in the α -synuclein gene (A53T) is associated with autosomal dominant PD in several families (1,2). There is also evidence suggesting a systemic defect in mitochondrial respiration in PD (3–7), possibly transmitted through the mitochondrial genome (8). This defect results in decreased activity of complex I of the mitochondrial respiratory chain (3) and links idiopathic and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism (9). MPTP causes degeneration of dopamine neurons through its metabolism by monoamine oxidase B (MAO B) to the neurotoxin 1-methyl-4-phenylpyridine (MPP+), which inhibits complex I activity (10). Other environmental factors may also influence the risk for PD (11–13). Endogenous neurotoxins, such as products of dopamine catabolism and/or isoquinolines, are also thought to potentially contribute to the pathogenesis of PD (14,15).

Neuropathologically PD is characterized by the loss of dopamine neurons in the substantia nigra pars compacta, with consequent severe reductions in the dopamine content of the striatum (16). Other areas of the brain (norepinephrine neurons in the locus ceruleus, cholinergic neurons in the nucleus basalis of Meynert) are also affected in PD, but to a lesser degree. The cause of the selective degeneration of dopamine neurons in PD is not understood at present. Several theories have been proposed to explain this selective loss. Data obtained from postmortem studies, as well as in vivo and in vitro experiments support a role of oxidative stress in the selective degeneration of dopamine neurons in PD.

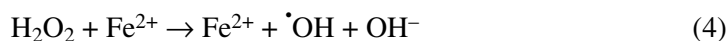
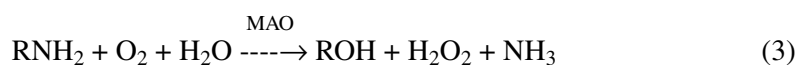
2. EVIDENCE OF OXIDATIVE STRESS IN PARKINSON'S DISEASE

Several observations point to the existence of conditions that either favor or are the result of oxidative stress in the substantia nigra of PD brains. Glutathione (GSH), one

of the major cellular antioxidants, has been shown to be decreased in the substantia nigra of PD brains (17,18). GSH is usually present in high concentrations in the brain where it acts as a reducing agent, removing hydrogen peroxide and maintaining the thiol groups of intracellular proteins (19).



The enzyme GSH peroxidase participates in the reduction of H_2O_2 (equation 1) and GSSG reductase in the regeneration of GSH from GSSG (equation 2). Decreased levels of cellular GSH in PD would create vulnerability to oxidative stress. Dopamine neurons may be particularly sensitive to GSH loss, in view of the production of H_2O_2 during the metabolism of dopamine by MAO (equation 3).



In the presence of iron, H_2O_2 can generate the highly cytotoxic hydroxyl radical (OH^\cdot) through the Fenton reaction (equation 4; for reviews on the subject *see refs. 14,20*). There are several reports of increased iron concentration in the substantia nigra of PD brains associated with melanin-containing dopamine neurons (21–25). Increased levels of iron, in combination with reduced GSH levels, would result in additional free radical generation, which could damage lipids, proteins, and nucleic acids. Increased lipid peroxidation, as well as DNA damage have been reported in PD brains (26,27).

Depletion of GSH can cause mitochondrial damage (28,29). A reduction in the activity of complex I of the mitochondrial respiratory chain, such as shown in PD substantia nigra (4), can also generate conditions of oxidative stress (30).

3. ROLE OF ASTROCYTES IN OXIDATIVE STRESS

Brain cells, which depend mainly on molecular oxygen for supply of energy, are equipped with safeguards for protection against oxidative stress. In addition to the antioxidant activity of the GSH-GSH peroxidase system described previously, there is also a high concentration of the antioxidant ascorbic acid in the brain (31) and ascorbic acid levels can be maintained in the brain under conditions of reduced supply (32). Other antioxidant defenses of the brain include catalase, which is present at relatively low concentrations (33), superoxide dismutase, α -tocopherol, and metal chelators such as metallothionins (34–36).

Glial cells appear to play a very important role in the protection of neurons from oxidative stress. Several *in vitro* studies have shown that the levels of GSH are higher in astrocytes than in neurons (37–40). In the brain, GSH concentration appears low in neuronal somata, whereas the neuropil, which includes glial processes, is enriched in GSH (41,42). Sagara and colleagues (39,43) have shown that glial cells, in contrast to

neurons, can effectively accumulate cystine from the extracellular space for use in GSH synthesis. Neurons, on the other hand, have a more efficient uptake for cysteine, which however is low in the plasma and culture media compared to cystine. Thus neurons depend on astrocytes for the export of cysteine into the extracellular fluid, which they can use for the synthesis of GSH (43).

Many studies have shown that glial cells have potent neurotrophic and neuroprotective activities toward dopamine neurons (44–46). In the presence of glial cells dopamine neurons become resistant to the neurotoxins MPP+ (47) and 6-hydroxydopamine (6-OHDA; 48,49). MPP+ uses the dopamine transporter to accumulate in the dopamine neurons (50,51) and then enters the mitochondria, where it inhibits NADH-ubiquinone oxidoreductase (complex I) activity of the electron transport chain (10,52). The interaction of MPP+ with complex I involves the generation of free radicals, which contribute to the irreversible inhibition of the enzyme complex and the toxic effects of MPP+ (53). 6-OHDA is a classic oxidative stress-inducing neurotoxin (54,55). Our studies showed that glial cells confer very powerful protection to dopamine neurons exposed to 6-OHDA (49). Glial growth was stimulated in mesencephalic cells in culture by treatment with basic fibroblast growth factor (bFGF). Treatment with bFGF in the presence of the mitotic inhibitor fluorodeoxyuridine (FUDR) completely abolished the protection against 6-OHDA by bFGF (Fig. 1A). When GSH levels were assessed in control and bFGF-treated cultures, it was found that in addition to the higher GSH levels, cultures containing glial cells were able to double their GSH content following 6-OHDA treatment (Fig. 1B). The upregulation of GSH levels following oxidative stress probably contributed to the protection from 6-OHDA toxicity. Inhibition of glial proliferation prevented the increase in the basal levels of GSH and also prevented the upregulation following 6-OHDA (Fig. 1C).

L-Dopa and dopamine are potent toxins to neurons when applied in culture (38,56–60). Toxicity is due to the autoxidation of the catecholamine moiety of the compounds (38) and is prevented by antioxidants (38,60). The presence of glial cells offers significant protection to the neurons against this oxidative stress paradigm (38,61). The mechanisms by which glial cells protect neurons against catecholamine toxicity are not well defined, but several properties of glial cells are consistent with a neuroprotective role. A study by Mena et al. (61) showed that glial conditioned medium was protective against L-dopa toxicity to dopamine neurons in culture, suggesting that glial cells may secrete antioxidants and/or other protective molecules. Both high and low molecular weight fractions (higher or lower than 10kD) of the glial conditioned medium had neuroprotective effects, as did directly applied glial cell line derived neurotrophic factor (GDNF) and GSH (61). Ascorbic acid, at concentrations present normally in the extracellular space (200 μ M), completely prevents L-dopa toxicity (58). Astrocytes can accumulate ascorbic acid, as well as its oxidized form, dehydroascorbic acid, from the medium (62–64) and could thus maintain the antioxidant status of brain cells.

Direct application of hydrogen peroxide to cultured neurons results in degeneration (65–67). The toxicity of hydrogen peroxide is attenuated when neurons are cultured in the presence of astrocytes (67,68). A study by Desagher et al. (67) showed that the degree of protection depended on the number of astrocytes and that significant protection against hydrogen peroxide could be achieved when one astrocyte was present per

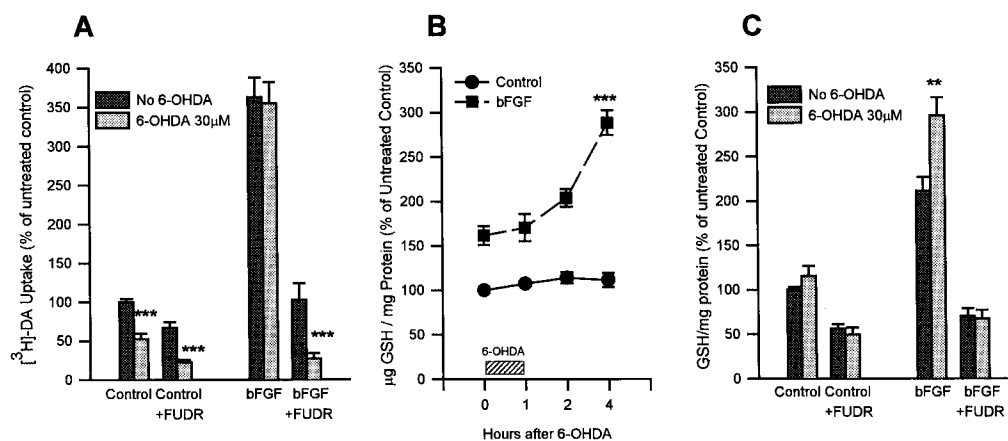


Fig. 1. Effect of glial cells on GSH levels and 6-OHDA toxicity in mesencephalic cultures. Glial growth was induced by treatment with 10 ng/mL bFGF. The mitotic inhibitor FUDR was used to prevent glial growth in the presence of bFGF. (A) [3 H]Dopamine uptake after treatment with 6-OHDA. Glial growth completely prevented the toxicity of 6-OHDA. (B) GSH levels in enriched neuronal cultures and in cultures treated with bFGF following exposure to 6-OHDA. bFGF-treated cultures had higher basal GSH levels and were able to upregulate GSH content after 6-OHDA. (C) Effect of inhibition of glial proliferation on GSH content. Treatment with FUDR completely prevented the increases in GSH levels caused by bFGF. ***Significantly different from corresponding control not treated with 6-OHDA $p < 0.001$. ** $p < 0.01$. Modified from (49).

20 neurons (67). Catalase and glutathione peroxidase are the major enzymes protecting from hydrogen peroxide toxicity. Glutathione peroxidase is exclusively localized in glial cells (astrocytes) in the brain (69), which probably explains the highly protective role of astrocytes against hydrogen peroxide toxicity. On the other hand Cu $^{++}$ /Zn $^{++}$ superoxide dismutase, which generates hydrogen peroxide from superoxide, is mainly localized within neurons (70,71), indicating the need for glial-neuronal interactions for protection from oxygen free radicals.

4. INFLAMMATION AND GLIAL CELLS IN PARKINSON'S DISEASE

Reactive microglia is found in the substantia nigra of PD patients (72,73), often associated with extraneuronal melanin granules and in some cases seen engulfing dopaminergic neurons (72). Proliferation of astrocytes (astrogliosis) is also present in the substantia nigra in PD (74). Whether these indices of an inflammatory reaction occur as a consequence of the continuous degeneration of dopamine neurons in the parkinsonian brain or they are playing a role in the process of neurodegeneration is not known. Recently, it has been proposed that activation of glial cells may contribute to the degeneration of dopamine neurons in PD (75). The authors hypothesize that activation of glial cells, caused by an as yet unknown agent, induces the synthesis and secretion of mediators of inflammation (cytokines, nitric oxide), which then interact with specific receptors and induce death of dopamine neurons (75). Several observations lend support to this hypothesis. Glial cells immunoreactive to interleukin 1 β (IL-1 β) and interferon- γ are present in the substantia nigra of PD patients (76). Tumor necrosis factor- α (TNF- α) is also expressed in glial cells in the substantia nigra of PD patients,

although it is essentially undetected in control brains (77). In addition, increased expression of TNF- α is found in the striatum and cerebrospinal fluid of PD patients (78). Furthermore, several other cytokines (IL-1 β , IL-2, IL-4, IL-6, transforming growth factor- α) known to be secreted by activated astrocytes and microglia are found in higher concentrations in the cerebrospinal fluid of PD patients (79).

5. MICROGLIA AND ASTROCYTES AS POTENTIAL CONTRIBUTORS TO NEURONAL TOXICITY

Microglia serve as the resident immune system of the brain and are essential for defense against inflammation and for wound repair (80). Upon activation they secrete a large variety of compounds including growth factors, cytokines, complement factors, lipid mediators, free radicals, and neurotoxins (*see* review by Minghetti and Levy; 80). Although microglia-derived compounds have been shown to enhance the survival of DA neurons in enriched neuronal cultures (81), activated microglia also secrete potential neurotoxins such as proinflammatory cytokines, glutamate, quinolinic acid, and nitric oxide (NO; 82).

As discussed earlier in this chapter, astrocytes play an important role in neuronal survival by releasing growth factors, providing important nutrients and GSH to neurons, sequestering and degrading excessive amounts of neurotransmitters and other potentially toxic molecules, and regulating ion homeostasis (*see* review by Tsacopoulos and Magistretti; 83). However, astrocytes may also contribute to neuronal degeneration. Two mechanisms potentially linking astrocytes to neurotoxicity are increased NO release and PLA₂ activation. Two major forms of nitric oxide synthase (NOS) have been identified. cNOS, present in the neurons, is a constitutive form of the enzyme activated by elevated intracellular Ca²⁺ levels (84). An inducible form (iNOS), is a Ca²⁺ independent enzyme expressed in cells related to the immune system and in astrocytes and microglia (85,86). cNOS activated by physiological agents causes release of NO, which is terminated within seconds or minutes, probably reflecting the return of intracellular Ca²⁺ to baseline. However, once iNOS is induced, NO release can continue for days (87). NO can cause oxidative damage and impair mitochondrial respiration (88,89) and it has been suggested that NO may play an important role in the pathogenesis of PD (90).

Astrocytes also contain phospholipase A₂ (PLA₂). The products of PLA₂-catalyzed reactions include free fatty acids such as arachidonic acid (AA). AA is one of the most important fatty acids in the brain, where it can serve as a precursor of prostaglandins, leukotrienes, and thromboxanes, biologically active substances important in signal transduction (91,92). However, it is believed that increased PLA₂ activity contributes to neurodegeneration in many disease states, including ischemia, Alzheimer's disease, schizophrenia, and head and spinal cord injury (*reviewed in ref. 93*). The cytokines TNF- α and IL-1 enhance the expression of PLA₂ in astrocytes (94). Increased PLA₂ activity can result in the accumulation of free fatty acids, which may initiate an "arachidonic acid cascade" resulting in the formation of ROS and membrane lipid peroxidation. In addition, activation of PLA₂ can lead to increased Ca²⁺ influx, which can result in apoptosis (95).

There is experimental evidence that, under certain conditions, glial cells may contribute positively toward neuronal toxicity. In a study using mesencephalic cultures,

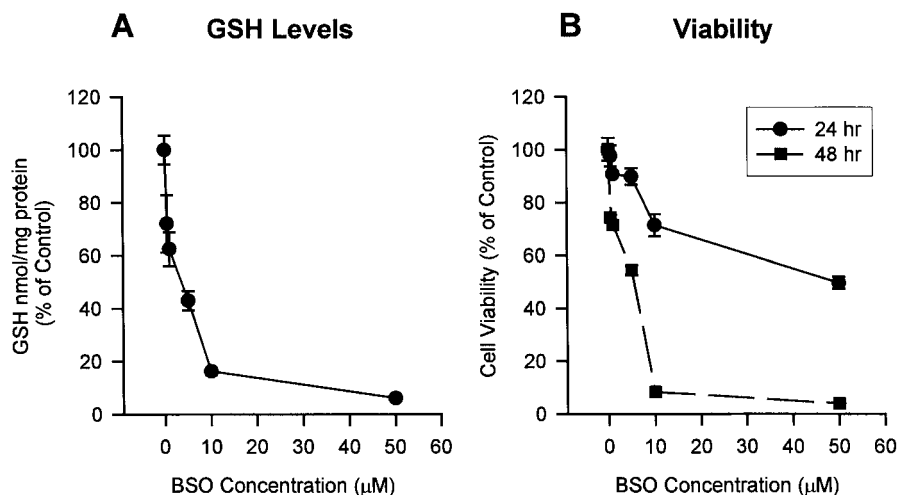


Fig. 2. Effect of treatment with increasing concentrations of BSO on GSH levels (A) and cell viability (B) in mesencephalic cultures containing glial cells. The growth of glial cells was stimulated by the use of serum in the medium. There was a dose dependent decrease in GSH levels 24 h after treatment with BSO. Viability also showed a dose dependent decrease following BSO, which was more pronounced 48 h after treatment.

Bronstein et al., (48) showed that a glial substrate protected dopamine neurons from 6-OHDA toxicity, but it promoted dopamine neuron degeneration in the presence of the endotoxin lipopolysaccharide (LPS). It is known that LPS causes activation of microglia and astrocytes. Inhibition of NOS activity by N^G-nitro-L-arginine methyl ester (NAME) completely prevented the effect of LPS, suggesting that NO, secreted by activated glial cells, was responsible for LPS toxicity (48). Similarly, a selective toxicity to dopamine neurons was observed in co-cultures of mesencephalic neurons and microglial cells, when they were stimulated with LPS or zymosan granules (96). Only the LPS-induced toxicity was blocked by inhibition of NOS, whereas zymosan toxicity resulted from protein kinase C activation (96). A selective increase in the survival of dopamine neurons in cultures treated with low concentrations of the antimetabolic agent cytosine arabinoside has also been attributed to the elimination of glial influences that promote neuronal apoptosis (97).

We have recently shown that glial cells are the mediators of neuronal cell death in mesencephalic cultures in a model of oxidative stress (40). Cultures were depleted of GSH using the GSH synthesis inhibitor L-buthionine sulfoximine (BSO). In cultures containing glial cells, treatment with BSO caused a dose dependent reduction in GSH content and cell death, which paralleled the loss of GSH but occurred with a time lag of about 24 h (Fig. 2). Similar reduction in GSH levels in enriched neuronal cultures caused no loss in cell viability (Fig. 3). The toxicity caused by GSH depletion was prevented by treatment with the antioxidant ascorbic acid, indicating that oxidative stress caused by the depletion of GSH was initiating events that led to cell degeneration. In addition to ascorbic acid, complete protection from toxicity was achieved by inhibition of lipoxygenase activity with nordihydroguaiaretic acid (NDGA), indicating an involvement of arachidonic acid metabolism in the toxic events (40). The fact that severe GSH

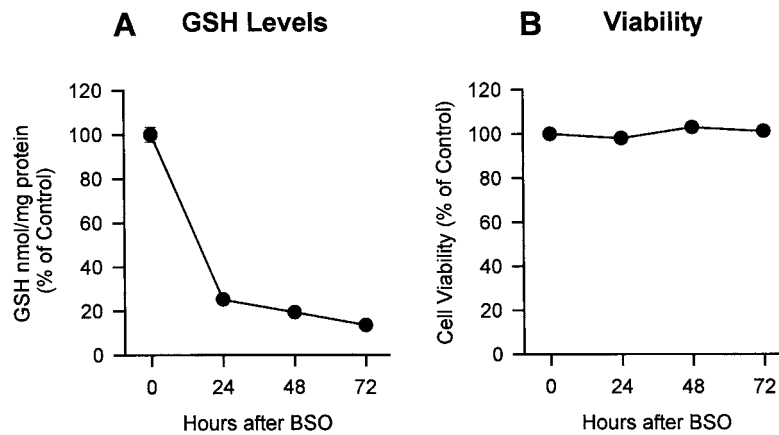


Fig. 3. Effect of BSO treatment on GSH levels and cell viability in enriched neuronal mesencephalic cultures. The growth of glial cells was prevented by the use of serum-free, chemically defined medium. Treatment with 50 μ M BSO resulted in increasing depletions of GSH over 72 h (A). However, no loss in cell viability resulted from this treatment at any time point (B).

depletion causes cell death only in the presence of glial cells suggests that oxidative damage resulting from loss of GSH may activate glial cells and this activation may lead to neuronal degeneration.

6. SUMMARY: GLIAL CELLS AS MEDIATORS OF PROTECTION AND TOXICITY IN PARKINSON'S DISEASE

The major neuropathological feature of PD is the severe loss of dopamine neurons of the substantia nigra pars compacta (76% loss; 98). Dopamine neurons in the pars lateralis, ventral tegmental area, and the peri- and retro-rubral regions are less affected (31–55% loss) and there is only minimal loss (3%) in the central gray (98). Even within the substantia nigra pars compacta there is differential sensitivity, with dopamine neurons on the dorsal part being less affected than those in the ventral tier (99). In a study of the distribution of astrocytes in the different parts of the substantia nigra of control human brains it was found that there was an inverse relationship between the density of astrocytes present and the extent of loss of dopamine neurons in PD (75). The authors propose that dopamine neurons in astrocyte-rich areas may be protected by neurotrophic factors secreted by glial cells or by the ability of astrocytes to catabolize dopamine and protect from free radical damage (75).

Microglia represent a substantial fraction of all glial cells in the brain (10–20%; 100) and the substantia nigra is the area with the highest concentration of microglia in the brain (101). Reactive microglia is observed in the substantia nigra of PD patients (72) suggesting the existence of an inflammatory response. Activated microglia and reactive astrocytes may play a role in neurodegenerative disorders through the secretion of proinflammatory cytokines, excitotoxins, and free radicals (80,102,103). The presence of proinflammatory cytokines, as well as increased levels of iNOS in glial cells in the substantia nigra in PD (75), suggest that an inflammatory response could be

contributing to the pathogenesis of the disease. If GSH depletion in the substantia nigra is indeed an early event in PD as suggested (104), one could hypothesize that oxidative stress-induced protein alterations may initiate a glial inflammatory reaction that could contribute to neurodegeneration. Our finding that depletion of GSH in mesencephalic cultures can induce neuronal damage, only in the presence of glial cells (40), supports this hypothesis.

In conclusion, glial cells provide protection and support neuronal survival. However, under special conditions, as during oxidative stress or inflammation, they might also secrete toxic compounds that promote neurodegeneration. A balance between neuroprotective and toxic influences is necessary for the maintenance of neuronal survival and function.

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Astrocytic Changes Associated with Epileptic Seizures

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1. INTRODUCTION

Throughout the central nervous system (CNS), most neurons are closely surrounded by astrocytes. In electron microscopic sections, astrocytes appear to encapsulate neuronal cell bodies and astrocytic processes reach close into the vicinity of synapses. This anatomical proximity gives astrocytes privileged access to the neuronal microenvironment and it is believed that astrocytes actively regulate the extracellular space surrounding neurons. Such regulation appears essential to ensure normal neuronal excitability, since even small changes in extracellular potassium (K^+), pH, or the accumulation of neurotransmitters can alter or compromise neuronal function. For example, recordings from hippocampal brain slices showed that modest elevations of extracellular K^+ results in hyperexcitability of hippocampal neurons (1), demonstrating properties that are reminiscent of epileptic seizures. Similarly, compromised astrocytic glutamate uptake, as observed for example in mice lacking astrocytic glutamate transporters, induces epileptic seizures in vivo (2). Thus, fine control of the microenvironment is essential for the maintenance of normal neuronal signaling. To accomplish this role, astrocytes express a number of transport systems, ion channels, and neurotransmitter receptors that are believed to jointly participate in the fine tuning of the neuronal microenvironment (for review, 3–11).

Epilepsy is characterized by spontaneous, synchronized discharges of neuronal populations. Episodes of such activity, also termed epileptic seizures, can originate from a number of conditions including acute trauma, brain oxygen deprivation, tumors, infections, or genetic abnormalities. In at least 50% of patients, no such causal factors can be identified that may have initiated the disease. Although epilepsy is generally considered to be a neuronal disease, astrocytes have long been recognized to show conspicuous morphological changes in conjunction with epilepsy. Thus, hypertrophic astrocytes (astrogliosis) were observed by Chasling in 1889 (12), and in 1927, Penfield (13) showed that reactive astrocytes are commonly present in brain regions exhibiting epileptiform activity. To the present date, however, there is no conclusive evidence that astroglial malfunction can induce or contribute to the generation of epileptic seizures. Nonetheless, accumulating evidence suggests that changes in the astrocytes associated

with epileptic seizures, may affect the composition of the neuronal microenvironment, which in turn may contribute to the long-term progression of the disease.

In this chapter, we will briefly review some of the most conspicuous morphological and physiological changes of astrocytes that have been associated with epileptic seizures, and will discuss the proposed influence that these changes may have on neuronal function in epilepsy. This is by no means a comprehensive review of the current knowledge of astrocytic changes in epilepsy but instead highlights some of the most evident changes that have been described. This chapter will review how reactive astrocytes contribute to neuronal hyperexcitability by means of chemical imbalances at synapses. We will further describe how reactive astrocytes may contribute to synaptic reorganization and neuronal loss, and how posttraumatic scar formation may contribute to hyperexcitability.

2. ASTROCYTIC CONTROL OF THE NEURONAL MICROENVIRONMENT AND CONTRIBUTIONS TO NEURONAL HYPEREXCITABILITY

For several decades, epilepsy has been studied in animal models or tissue slice models of epilepsy in which seizures were chemically induced. Essentially all these acute models induce a chemical imbalance that alters synaptic transmission in ways that induce repetitive and synchronous neuronal discharges. The simplest of these models chronically depolarizes neurons by bathing slices in elevated K^+ (1). Alternatively, hyperexcitability can be induced by interfering at the level of synaptic communication between neurons, as for example in the bicuculline model of epilepsy, in which gamma-aminobutyric acidergic (GABAergic) inhibition is reduced by blockade of $GABA_A$ receptors (14,15).

In these models, ionic imbalances cannot only induce seizures, but more importantly, the resulting neuronal excitability can itself lead to increases in extracellular K^+ concentration ($[K^+]_o$) and neurotransmitter concentrations. For example, a single action potential can transiently change $[K^+]_o$ by 0.75 mM. Repetitive stimulation can result in increases in excess of 10 mM (16). Similarly, it is estimated that glutamate concentrations rise to hundreds of micromoles following intense neuronal activity. These changes are transient and ion homeostasis is reestablished within seconds by the combined activity of both neuronal and glial transport systems and ion channels. Any perturbation of the ion homeostatic transport processes are expected to greatly alter neuronal signaling and have long been speculated to contribute to epileptic seizures. Maintenance of extracellular K^+ and glutamate has been the focus of numerous studies aimed at delineating the possible contributions of astrocytes to epileptic seizures, and these studies will be discussed henceforth.

2.1. Control of Extracellular K^+ by Astrocytes

The spatial buffer hypothesis for K^+ , dating back to the early physiological studies by Kuffler and colleagues (17) in the nervous system of the leech, has been extensively reviewed (18–25). This hypothesis proposes the diffusional uptake of K^+ by glial cells and its subsequent spatial redistribution to areas where K^+ concentrations are low. In theory, any elevation of $[K^+]_o$ in the extracellular space (ECS) following neuronal discharge can be dissipated via different mechanisms:

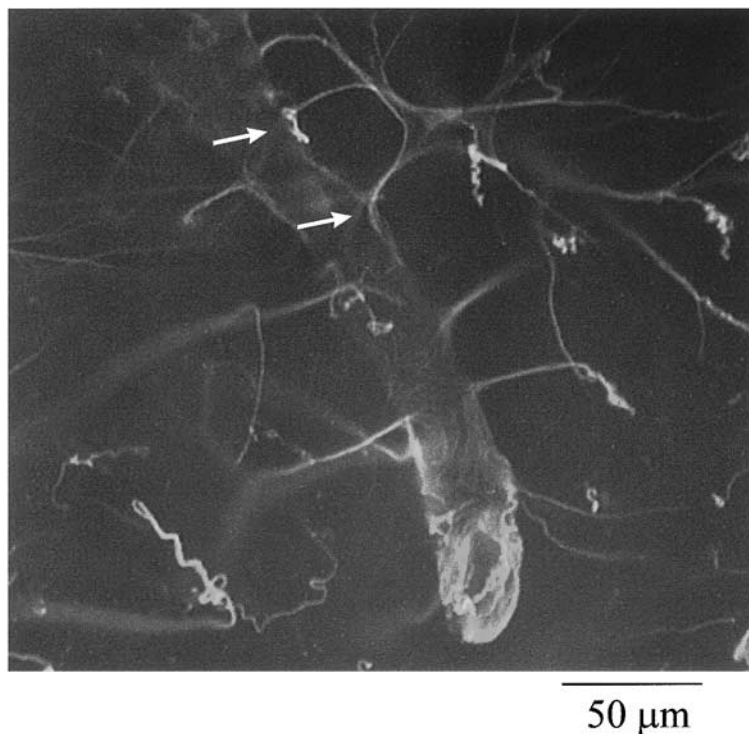


Fig. 1. GFAP antibodies stain astrocytes in CA1 rat hippocampus. Astrocytes display endfeet (white arrows) contacting blood vessels. The staining was performed 14 d after intraperitoneal kainic acid injection.

1. K^+ can simply diffuse through the ECS;
2. K^+ can enter astrocytes via channels or pumps, and
3. K^+ can be taken-up into neurons.

On theoretical grounds, the diffusional dissipation of K^+ through the extracellular space appeared insufficient and has since been rejected experimentally (26,27). However, excellent support remains for the notion that the majority of K^+ enters glial cells rather than neurons. K^+ uptake may occur through ion channels, co-transporters, or the Na^+/K^+ -pump and, indeed, evidence exists to implicate all of these mechanisms. For example, in retinal glial cells, K^+ uptake through inwardly rectifying K^+ (Kir) channels, which are abundantly expressed by astrocytes and retinal glial cells, has been demonstrated to be a key mechanism in controlling activity-dependent changes in K^+ (28,29). In cultured astrocytes, a significant uptake also occurs via the $Na^+/K^+/2Cl^-$ co-transporter (for review, 30.) Activity of the latter results in a net influx of water leading to astrocyte swelling (27,31–33), which is a consistent feature in hyperexcitable tissue. Unquestionably, uptake into both astrocytes and neurons may occur via the Na^+/K^+ -ATPase and at least one study has implicated this pathway in the K^+ uptake into axons in optic nerve *in situ* (34).

Intuitively, neuronal uptake of K^+ would be the most logical mechanism, as K^+ ultimately needs to be replenished to the cell from which it is released. Glial K^+ uptake, however, may have some distinct advantages over neuronal uptake. Most notably, the

gap-junction coupling of glial cells allows them to share a large common cytoplasm and promotes the rapid dilution of any ions taken up (35). Moreover gap junctions also facilitate the redistribution of K^+ within the glial syncytium, specifically the transport of K^+ away from the site of uptake, and toward astrocytic endfeet onto blood vessels, which are believed to function as K^+ release sites into the blood stream (Fig.1). Consistent with this theory, clustering of K^+ channels has been observed on astrocytic endfeet (28).

Although astrocytes were long considered to be exclusively permeable to potassium ions, accumulating evidences show that they express a whole series of voltage-activated (VA) ion channels for sodium (Na^+), calcium (Ca^{2+}), and chloride (Cl^-) (for review, 5,6,36,37). Any local increase in K^+ will thus depolarize the astrocytic membrane possibly activating these voltage-dependent channels. How and if VA ion channels contribute to K^+ buffering is, as of yet, not clear. It has been proposed that entry of Na^+ ions through VA Na^+ channels is required to fuel the astrocytic Na^+/K^+ pump (38).

The notion that a failure to buffer K^+ may underlie epileptic seizures has been around since the late 50s though various studies employing different approaches have come to very divergent conclusions. The concept of high K^+ as a cause epilepsy came from early work with ion-selective microelectrodes demonstrating that physiological increases in $[K^+]_o$ reach a ceiling of about 10 mM (16). When this level is reached, the control mechanisms involving neuroglial interactions breakdown (19,21–23), resulting in spreading depression (39) during which $[K^+]_o$ increases up to 40 mM (40,41) and extracellular Na^+ , Cl^- and Ca^{2+} concentrations decrease (42–45). During in vivo seizures in cats, $[K^+]_o$ also increased from 3 mM to 10–12 mM (40,46–48). These data lead us to ask the two following questions.

2.2. Are Increases in K^+ Alone Sufficient to Induce Seizures?

Traynelis and Dingledine (1988) reported that bath application of 8 mM K^+ can trigger seizures in the CA1 region of the hippocampus. In 30 to 60 seconds prior to seizure onset, both astrocytes and neurons in CA1 depolarize and there is a concomitant shrinkage of the extracellular space. Seizures can be blocked by the addition of hyperosmotic medium or by application of NMDA receptor antagonists (49,50). It is thought that elevated K^+ depolarizes neurons thereby moving them closer to the threshold for spiking (51). However, elevations of K^+ trigger a whole cascade of events, outlined below, which may contribute to the hyperexcitability.

1. In astrocytes, K^+ influx through transport systems leads to the net influx of K^+ , Cl^- , and water resulting in cell swelling and shrinkage of the extracellular space (20,21,31,32,52–54). These effects are also observed during neuronal hyperactivity (43,55–57). The reduction of extracellular space will alter the concentration of extracellular Na^+ and Cl^- ions (57,58) and may increase neuronal synchronization due to ephaptic communication (59).
2. Activation of VA ion channels in astrocytes leads to influx of Ca^{2+} and Na^+ . VA sodium channels are necessary to fuel the Na^+/K^+ ATPase (38) required to extrude K^+ from astrocytes during high neuronal activity (60,61). Astrocytic calcium influx may contribute to the reported decrease in $[Ca^{2+}]_o$ in the interstitial spaces from 1.2 to 0.7 mM reported during seizures (57,62,63). The importance of these changes in Ca^{2+} are not entirely known, but several studies have shown that perfusion of hippocampal slices with low Ca^{2+} is sufficient to induce seizures (64,65). Interestingly, several clinically used anticonvulsants including phenytoin, valproate, and ethosuximide attenuate or

inhibit the high potassium-induced Ca^{2+} increases in astrocytes via blockade of astrocytic VA Ca^{2+} channels (36,66,67).

3. Depolarization of astrocytes induces the release of aspartate and glutamate by reversal of glutamate transporters, the contribution of which to epilepsy is further discussed below. Briefly, any increases in extracellular glutamate would further activate both neuronal and glial glutamatergic receptors. Moreover, glutamate can also induce cell swelling (68–70), which in astrocytes would further enhance glutamate release (71,72). Astrocytic swelling appears to be a key element in this cycle and can be mimicked by hypo-osmotic conditions that also induce seizures (for review, 73). Further evidence that astrocyte swelling is of crucial importance to these events is the finding that furosemide, a chloride co-transporter inhibitor can inhibit seizure activity in hippocampal slices (74).

2.3. Are Astrocytes Responsible for the K^+ Imbalance?

The participation of posttraumatic reactive astrocytes in K^+ disturbance and epileptogenesis was first proposed by Pollen and Trachtenberg (75), and it has been recently reported that a reduction of K^+ uptake in glial cells can induce epileptiform activity *in situ* (76). In this study, the authors recorded hippocampal astrocytes while monitoring network electrical activity. When they blocked astrocytic potassium channels, in particular Kir channels by cesium, stimulation-induced $[\text{K}^+]_o$ increased and synchronous interictal bursting occurred. Interestingly, we reported that these channels were lost in some reactive astrocytes recorded in human epileptic tissue, *in situ* (77). In this study, astrocytes in the epileptic foci also had a depolarized membrane potential because of the loss of Kir channels. This result is in agreement with the early work of Picker et al. who showed that reactive glial cells from patients with intractable, focal epilepsy were less permeable to K^+ than normal glial cells (78). As a result, they proposed that gliotic astrocytes associated with epileptic seizure foci are intrinsically less capable of buffering K^+ than normal astrocytes.

These results, however, differ from the conclusions drawn by two earlier studies (79,80). Burnard et al. (1990) reported no changes in the resting membrane potentials of reactive astrocytes in kainate-lesioned hippocampal slices, and Glötzner (1973) found that in epileptic foci induced by aluminum hydroxide, feline glial cells could transport even more K^+ away from sites of K^+ release than they do under normal conditions. It is possible that different models of epilepsy could account for this discrepancy. In addition, Glötzner only reported cells displaying a resting potential less than -50 mV although lower potentials were encountered more frequently in gliotic astrocytes. Hence, these studies may have only reported on a subpopulation of astrocytes. In fact, we have reported two populations of reactive astrocytes in epileptic tissue, one with depolarized resting potentials and no Kir channels and another one with increased passive K^+ currents and more hyperpolarized potentials (77).

The information reported above, which are summarized in Figure 2 provides little evidence that astrocytes directly initiate seizures. However, they strongly suggest a more indirect participation of astrocytes in the generation of seizures, primarily due to a compromised ability to buffer K^+ within seizure foci.

2.4. Astrocytic Control of Glutamate and Potential Consequences for Epilepsy

Glutamate is the major excitatory neurotransmitter in the CNS. The threshold for activation of glutamate receptors at the postsynaptic membrane is only marginally

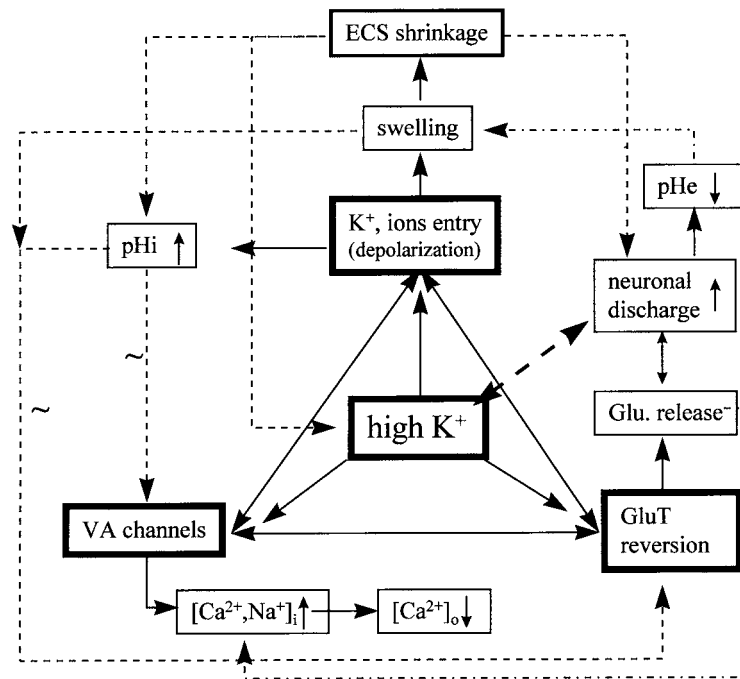


Fig. 2. This schematic displays a hypothesis of how high potassium might initiate hyperexcitability and amplify seizure activity.

above the normal levels of free glutamate in the interstitial space ($1\ \mu\text{M}$) (81). Moreover, even a transient exposure of neurons to elevated glutamate levels has been shown to lead to widespread neurotoxicity (82–84). It is thus essential to keep glutamate concentrations in the interstitial space tightly controlled in order to assure normal synaptic transmission and to avoid excitotoxicity.

Glutamate has been implicated in the pathogenesis of epilepsy (85–87) and altered levels of glutamate and aspartate have been reported in patients with epilepsy by microdialysis (88–91). Moreover, a number of experimental animal models for epilepsy present with increased interstitial glutamate levels (92–98) supporting the notion that increases in interstitial glutamate may contribute to epilepsy. Increased glutamate levels may be attributed to increased activity of glutamatergic neurons. Yet, under normal physiological conditions, glutamate levels in the brain are tightly controlled by both neuronal and glial uptakes, both expressing Na^+ -dependent glutamate transporters. Astrocytes express primarily GLT1 (also called Excitatory Amino Acid Transporter 2, EAAT2) and GLAST (or EAAT1), whereas neurons appear to express primarily EAAC1 (or EAAT3) (99–102). The implication of astrocytic glutamate transporters in epilepsy is supported by recent knockout studies in which the selective loss of GLT1 *in vivo* leads to markedly increased interstitial glutamate levels that were associated with excitotoxicity (103). In addition, mice lacking the glutamate transporter, GLT-1, exhibited epileptic seizures (2). Neither seizures nor excitotoxicity was observed in EAAC1 knockouts. Thus if compromised transporter function is involved in human epilepsy, it appears most likely that the astrocytic glutamate transporter function is impaired. Changes in the expression levels of astrocytic glutamate trans-

porters are reported in some studies of experimental epilepsy. Thus, in kindling-induced epilepsy in rats, the astrocytic glutamate transporter, GLAST, was downregulated in the piriform cortex/amygdala but not in the hippocampus and GLT-1 was unchanged (104,105). In genetically epilepsy-prone rats, GLT-1 and EAAC1 displayed differences in mRNA levels but there was no reported obvious changes in the expression of the corresponding proteins (106).

Impairment of glutamate transport may not require any changes in the expression levels of the transporter but may be due to functional modulation of uptake. Interestingly, all the cloned glutamate transporters show a strong dependence on transmembrane voltage and gradients for Na^+ , K^+ , and protons (H^+) (for review *see* refs. 101,102,107–109). Thus, the above mentioned accumulation of $[\text{K}^+]_o$ during seizure activity would be sufficient to greatly reduce glutamate uptake and indeed may even lead to nonvesicular glutamate release through depolarization-induced reversal of the astrocytic glutamate transporters (110–112). A number of other factors have been shown to decrease the glutamate transporters expression and/or activity (for review, 101). For example, glutamate transport is inhibited by arachidonic acid (113), which is released by neurons during seizure activity (114). Oxygen free radicals and in particular nitric oxide or its metabolites which are formed following NMDA receptor activation, can similarly inhibit astrocytic glutamate transport (115–119). A number of cytokines that have been shown to be activated in animal models of epilepsy are also potent inhibitors of astrocytic glutamate transport (119,120). Finally both cyclic adenosine-monophosphate (cAMP) (121,122) and protein kinase C (PKC) (123,125) can modulate glial glutamate transporters, and both have been shown to be activated following neurotransmitter receptor activation. Thus collectively, these data suggest that hyperexcitability would, in many ways, negatively feed back on astrocytic glutamate transport, thereby enhancing interstitial glutamate levels.

When one considers the role that astrocytes play in the metabolism/catabolism of glutamate, the contribution of glutamate uptake by astrocytes to the formation of seizures, becomes even more complex. The neuronal metabolism of glutamate depends on the astrocytic supply of glutamine (126,127) and tricarboxyl acid (TCA) intermediates (*see* 128), which are synthesized by the astrocytic enzymes, glutamine synthetase (GS) (129,130) and pyruvate carboxylase (131–133), respectively. Concerning the expression of GS in experimental epilepsy, the data are controversial and vary with species and epileptic model. In the Mongolian seizure prone-gerbils, there was a deficiency in cerebral GS (134), and likewise, in the genetically epilepsy prone-rat, GS activity was significantly lower than in control brain (135). In experimental models of FeCl_2 -induced focal and kindled amygdaloid seizures, there was an increase and a decrease in GS activity, respectively (136). The authors concluded that increased GS activity is associated with submaximal seizure development, whereas decreased enzyme activity is characteristic of mature, chronic seizures such as those of kindling or epilepsy-prone animals. Concerning the pyruvate carboxylase, no changes have been reported in epilepsy. However, the activity of another enzyme, the aspartic acid aminotransferase, involved in the aspartate metabolism in both astrocytes and neurons, is increased in human epileptic cortical tissue (137).

Another aspect of neurotransmitter accumulation in the synaptic cleft, is the activation of their receptors on the astrocytic membranes. Astrocytes possess receptors for

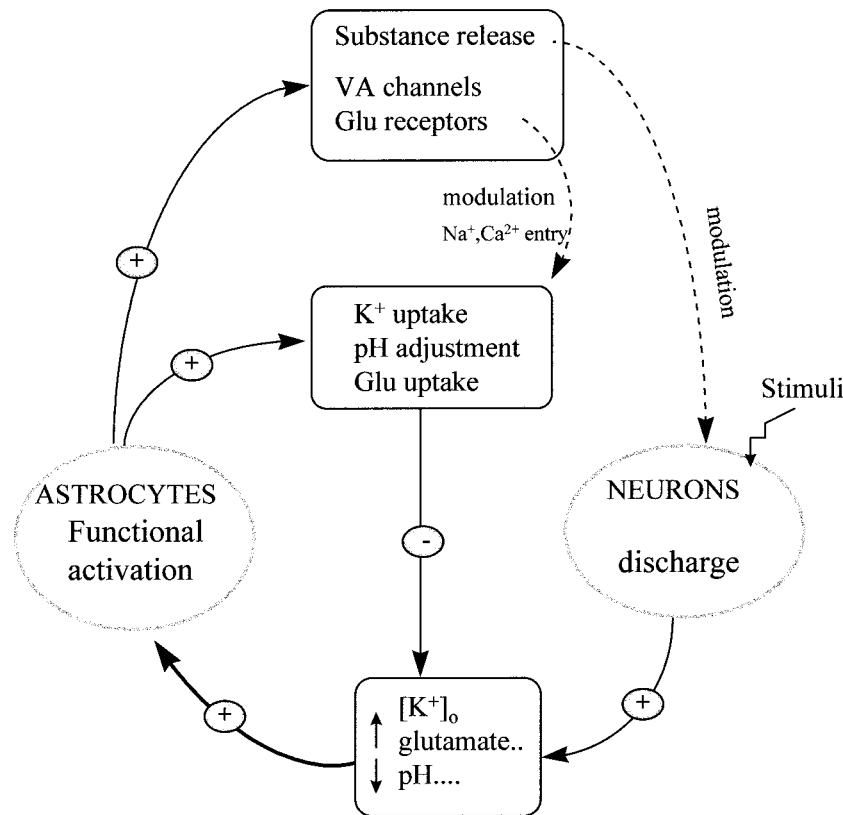


Fig. 3. Simplified and summarized schematic describing the regenerative cycle leading to epileptic seizures.

almost every neurotransmitter present at synapses (7–10). Although the role of these receptors is currently unclear, it has been proposed that they primarily allow astrocytes to sense synaptic activity. It is now clear that activation of glutamate receptors in astrocytes can induce calcium increases and spreading Ca^{2+} waves (discussed in Subheading 2.5.).

The diagram in figure 3 summarizes the activation of glial cells following neuronal discharge and their possible role in restoring normal extracellular conditions suitable for proper neuronal function. This cycle is oversimplified since not only glutamate is released by neurons but many other neurotransmitters can be involved depending on the CNS region. Nevertheless, it sets the starting point from which to discuss the role of neuron-glia interactions in the context of epilepsy.

2.5. Astrocytic Ca^{2+} Waves Triggered by Glutamate

An increasing amount of evidence suggests that neuronally released transmitters and peptides can induce Ca^{2+} responses in astrocytes, and may even lead to spreading Ca^{2+} waves within the glial syncytium. This exciting area of glial physiology is still poorly understood but provides “food for thought” as to the design of future studies aimed at delineating the role of astrocytes in epilepsy. Many transmitters that are released by

enhanced neuronal activity, can produce increases in astrocytic intracellular calcium levels (7–9,138,139). For example, glutamate receptor induced depolarization triggers influx of Ca^{2+} through VA Ca^{2+} channels (37,140–144). These channels are upregulated in kainic acid induced-epilepsy (145). Astrocytic muscarinic acetylcholine receptors are likewise upregulated after amygdala kindling (146) and induce increases in Ca^{2+} via PKC activation. Any rise in astrocytic Ca^{2+} would have marked and immediate effects on K^+ buffering, astrocyte swelling, and glutamate release, each further enhancing neuronal excitability (for more details about Ca^{2+} roles, see 147). In addition, these Ca^{2+} rises spread in the form of slowly traveling astrocytic calcium waves (35,139,147–151). The spread of these waves may be facilitated in epileptic tissue as astrocytes from epileptic patients demonstrate increased gap junctional coupling (152,153). The functional implications of Ca^{2+} rises are many-fold and include those typically associated with this important messenger molecule. In the context of astrocyte function in epilepsy, it is easy to envision how increases in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) may affect the reactive phenotype, stimulate glycogenolysis, and modulate gene expression. Indeed, an induction of transcription factors and immediate-early genes by increases in $[\text{Ca}^{2+}]_i$ has been demonstrated in astrocytes (for review see refs. 139,154,155). These may subsequently induce the long-term changes in gene expression typical of reactive astrocytes that characterize the glial scar often associated with seizure foci.

Overall these data suggest that under seizure conditions astrocytes appear to lose their tight control over extracellular glutamate levels that they maintain in the normal brain. Although this may not be causative for the disease, it certainly may be an important contributing factor to the long-term pathology associated with epilepsy. Increases in Ca^{2+} influx into astrocytes and the spreading of Ca^{2+} waves, may induce expression of a reactive astrocytic phenotype.

3. MORPHOLOGICAL CHANGES INVOLVING ASTROCYTES

In the hippocampi of epileptic patients with medial temporal lobe epilepsy, neuronal reorganizations occur in conjunction with astrocytic hypertrophy. Neuronal cell death has been documented in the hippocampal CA3 (Ammon's horn) and dentate hilus (12,156,157). In addition, mossy fiber sprouting e.g., the sprouting of granule cell axons into the inner molecular layer of the dentate gyrus, and the formation of recurrent excitatory circuits by innervation of granule cell dendrites is a conspicuous feature (158–160). These morphological changes can be partially induced in two experimental models, namely kindling of limbic structures (161,162), and the administration of kainic acid (163–165).

3.1. Morphological Changes in Astrocytes

In medial temporal lobe epilepsy, a glial scar is frequently associated with the epileptic seizure focus, and these scars resemble those seen after acute brain injury. Surgical removal of this scar is essential to surgically cure patients from seizures. These scars contain astrocytes that are characterized by hypertrophy and cytoskeleton changes, of which accumulation of intermediate filaments is most conspicuous. The principal protein constituting astrocytic intermediate filaments is glial fibrillary acidic protein (GFAP) (166) and its intense immunoreactivity is commonly used to identify astrogliosis (Fig. 4, 5). Such

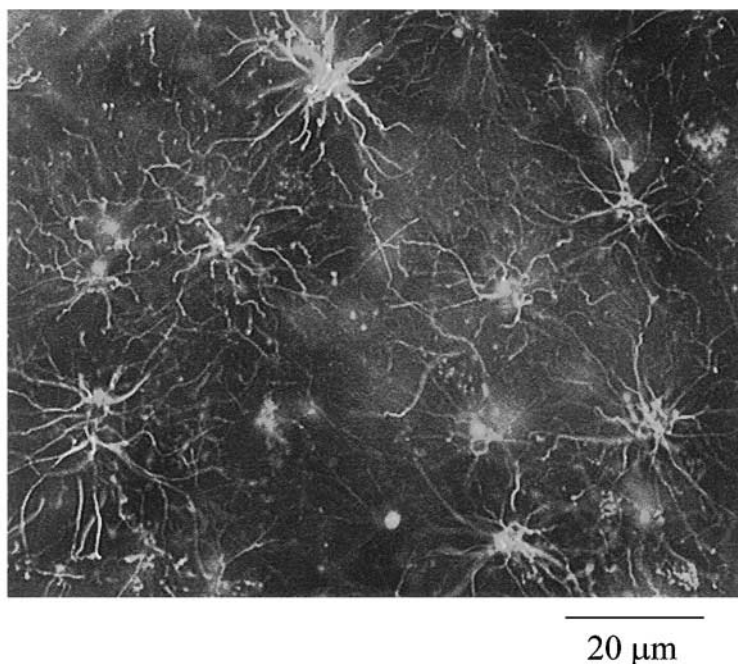


Fig. 4. Astrocytes in slices from a patient biopsy stained intensively for GFAP. The patient was operated for intractable mesio-temporal lobe epilepsy.

astrocytes are termed reactive astrocytes and include at least some proliferative cells (167,168). Interestingly, two distinct populations of reactive astrocytes, one nonproliferative and another proliferative have been observed in the kainic acid-induced seizure model (for review, 163,169,170). In the hippocampal region associated with neuronal degeneration (CA3 and hilus), astrocytes proliferate whereas in regions with only axonal degeneration (e.g., molecular layer) astrocytes do not proliferate (163,167,168). It has been shown that hypertrophy of astrocytes can result from abnormal neuronal activity and in the dentate gyrus leads to increased GFAP mRNA (171,172) and GFAP protein levels (169,173). Astrogliosis seems to be an early event that precedes neuronal degeneration. By contrast, astrocytosis (astroglial proliferation) occurs once the degeneration of neurons has been initiated (167,168,174,175).

3.2. Trophic Role

The role that these glial scars play in the vicinity of epileptic seizure foci is not entirely clear. In addition to filling spaces left vacant by dead neurons, astrocytes might release neurotrophic factors (NTF) and cytokines, the levels of which are significantly altered in epileptic tissue (176–182, for review *see ref. 183*). It has been postulated that the sprouting of mossy fibers in conjunction with seizures involves seizure-induced expression of neurotrophic genes (184,185). Increased levels of glial cell-line derived neurotrophic factor (GDNF) released by astrocytes have been reported (186,187), suggesting a potent role for astrocytes in synaptic reorganization (188,189). Other cytokines like IL-6 and S100 β display increased levels in cerebrospinal fluid from epileptic patients and in human epileptic cortical tissue (190,191), respectively. Since

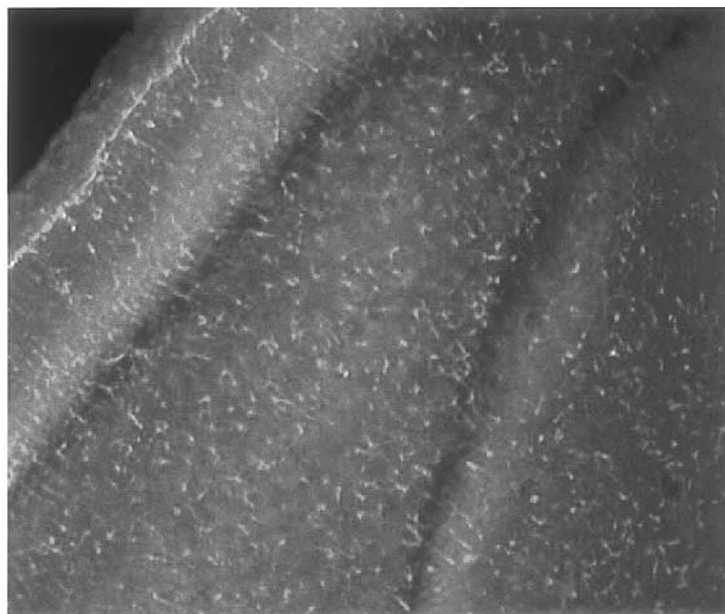
200 μm

Fig. 5. Distribution of GFAP-immunoreactive astrocytes in the hippocampus (dentate gyrus) of a rat, 14 d after intraperitoneal injection of kainic acid.

cytokines are trophic factors for neurons, enhancing neuronal survival and neurite outgrowth (192–195), the over-expression of cytokines by astrocytes may have deleterious consequences, including excessive growth of neurites. In addition, a release of NTF and other cytokines can have an autocrine effect on astrocytes resulting in morphological changes and enhanced proliferation (196).

Thus, neuronal reorganizations that occur in the context of epileptic seizures, appear to occur in conjunction with gliosis, and some of the reorganization may be greatly influenced by trophic factors and cytokines released by reactive astrocytes.

An active role of the scar tissue which surrounds the area of cortical damage in epileptogenesis was proposed by Penfield in 1927. He considered the scar tissue as a structural basis of epilepsy leading to the “irritation” of neurons. This concept was also stated by Ward in 1961 (*see* 197), who proposed that astrogliosis caused a mechanical deformation of dendrites, resulting in neuronal membrane depolarization and hyperexcitability. Although this reasoning cannot be discounted entirely, more plausible explanations may exist for neuronal hyperexcitability that include changes in ion homeostasis in the immediate vicinity of the seizure focus. This topic has already been discussed in detail previously.

4. CONCLUSION

Surgical management of intractable epilepsy includes, if possible, the removal of the glial scar associated with the epileptic seizure focus. Much evidence suggests that astrocytes within epileptic tissue show markedly altered abilities in K^+ buffering and in

their ability to handle extracellular glutamate. Taken together, these data suggest that astrocytes surrounding epileptic seizure foci may be ill equipped to deal with the enhanced neuronal activity and may thus contribute to the progression of epileptic pathology. The release of neurotrophic factors and cytokines could further contribute to some of the morphological rearrangements commonly observed in epilepsy, and thus, may expand the role astrocytes play beyond that of extracellular space homeostasis. Further studies will hopefully elucidate further evidence that astrocytes are indeed protagonists in this disease.

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Synaptic and Neuroglial Pathobiology in Acute and Chronic Neurological Disorders

Lee J. Martin

1. INTRODUCTION

Pathology at the molecular and cellular level is the fundamental basis of disorders that effect the brain or spinal cord of humans. A pathophysiological event can target specific molecules and groups of cells, causing neuronal dysfunction and degeneration and resulting in acute and chronic neurological and behavioral disabilities ranging from memory loss to paralysis. For example, Alzheimer's disease (AD) is associated with loss of forebrain neurons and the formation of brain lesions consisting of abnormal deposits of glial- or -neuronal generated amyloid β protein, possibly in response to gene mutations, synaptic perturbations, oxidative stress, or neuronal cytoskeletal defects (1–5). The degeneration of motor neurons in amyotrophic lateral sclerosis (ALS) is associated with impairments in glutamate reuptake by astroglia (6,7), gene mutations in superoxide dismutase-1 (8), and abnormal apoptosis of motor neurons that appears to be mediated by programmed cell death mechanisms (9,10). The nerve cell damage in adults and children that have experienced cardiac arrest, asphyxiation, strokes, and head or spinal cord trauma may be caused by failure of astroglial glutamate transport, excessive stimulation of glutamate receptors, abnormal activation or impaired function of intracellular signaling pathways, toxic generation of free oxygen radicals, and structural damage to target molecules within selectively vulnerable populations of neurons (11). Epidemiological studies reveal the impact that neurodegenerative disorders have on our society. For example, AD affects ~4 million adults (most are >65-yr-of-age) and is the fourth-leading cause of death in the United States, accounting for >100,000 deaths annually (12). ALS affects approx 30,000 Americans (4–6 people in 100,000) (13). Stroke is the third leading cause of death in industrialized populations and is a major cause of long term neurological disability (14).

No cures or therapies (e.g., synthetic drugs or biological factors) are available yet that can prevent the degeneration of neurons in the human brain and spinal cord and that can improve the quality of life for individuals with these neurological disorders. The current inability to effectively and rationally manage and treat individuals with these nervous system abnormalities is due to insufficient information on how neurons and glia become dysfunctional or degenerate and the synaptic and molecular mecha-

nisms for this pathobiology. This chapter will focus on synaptic and neuroglial perturbations in two broad settings of neurodegeneration: Acute neuronal cell death after cerebral ischemia and chronic, progressive neuronal degeneration that occurs in age-related disorders of the nervous system, such as AD and ALS. Treatments for these disorders will be forthcoming eventually through a more complete understanding of the normal cellular and molecular organization of the nervous system and of cellular and molecular neuropathology.

2. ORGANIZATION OF THE CENTRAL SYNAPSE

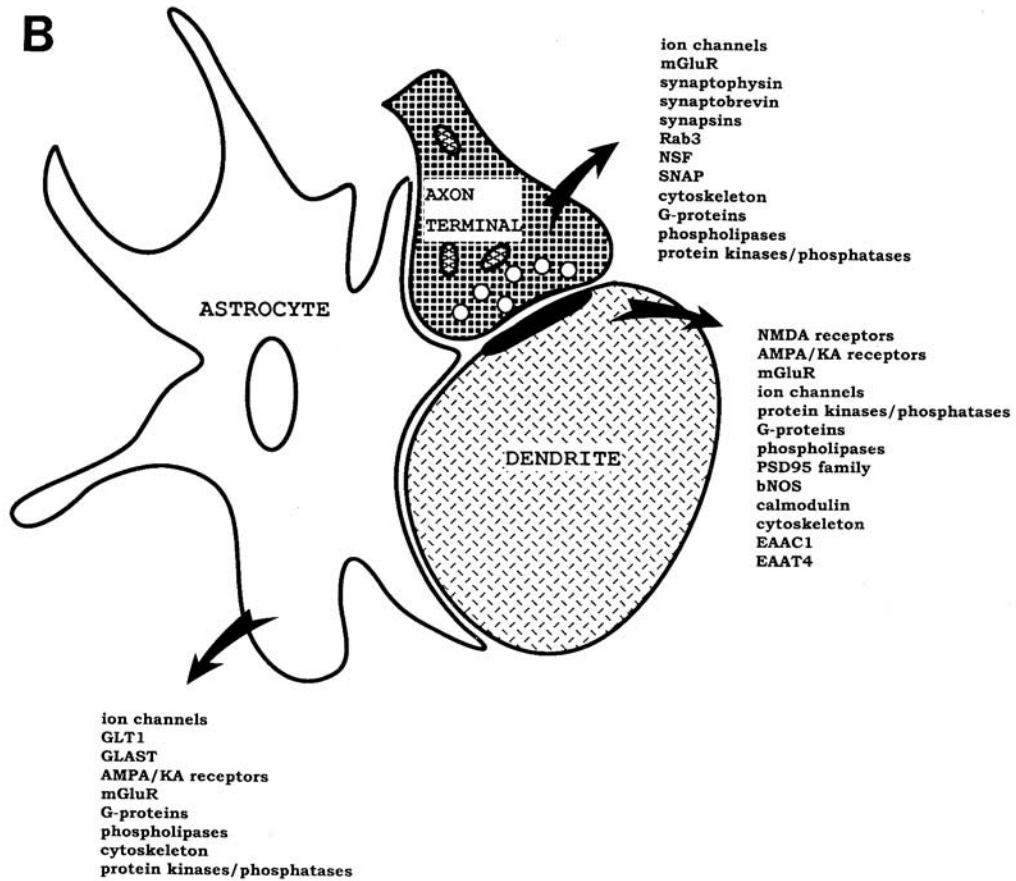
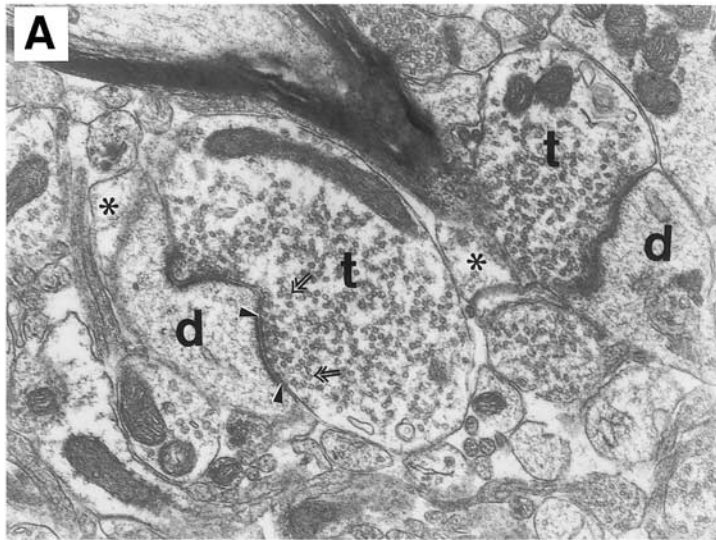
2.1. *Structural and Molecular Design of the Synapse*

Synapses are the principal units of intercellular communication in neural circuits. The structural organization of a central nervous system (CNS) synapse consists of a presynaptic component (the axon terminal), a synaptic cleft (~20–40 nm in width), a postsynaptic component (a neuronal cell body or process), and an astroglial sheath (Fig. 1). Each of these components has its own elaborate structural organization and molecular specifications. The presynaptic and postsynaptic membranes have thickenings on their cytoplasmic surfaces (Fig. 1), and these specialized membranes together with the synaptic cleft form the synaptic junction. These membrane specializations are the presynaptic grid or active zone and the postsynaptic density.

The presynaptic nerve terminal (Fig. 1A), containing several hundred neurotransmitter vesicles, is the principal site of regulated release of excitatory or inhibitory neurotransmitters. A complex ensemble of proteins (Fig. 1B) within the presynaptic nerve terminal functions in exocytosis of neurotransmitter-containing vesicles (15). Synaptic vesicles dock at the active zone. Specific proteins function in neurotransmitter vesicle docking, priming, fusion, and exocytosis and endocytosis. This process is called the synaptic vesicle cycle (15).

Dendrites receive the vast majority of excitatory synapses in the mammalian CNS (Figs. 1 and 2). Like the presynaptic terminal, the postsynaptic element contains an equally staggering array of proteins that function in inter- and intracellular signaling. These proteins are neurotransmitter receptors (ionotropic and metabotropic receptors), ion channels (Ca²⁺, Na⁺, K⁺, and Cl⁻ channels), and other signal transduction molecules, including protein kinases, protein phosphatases, heterotrimeric GTP-binding proteins (G-proteins), phospholipases, nitric oxide synthase (NOS), calmodulin, and cytoskeletal proteins (Fig. 1B). At the postsynaptic site, the postsynaptic density is an

Fig. 1. (Right) Organization of a central nervous system synapse (A) Electron micrograph illustrating the synaptic complex. Two presynaptic axon terminals (t), containing numerous neurotransmitter vesicles (arrows) which are ~50 nm in diameter, form synaptic junctions with dendrites (d). The dark thickening at the dendritic side of the junction is the postsynaptic density. The synaptic cleft of one junction is identified (arrowheads). Astroglial processes (asterisks) ensheath the synaptic complex. (B) Simplified synaptic organization in forebrain as represented by an presynaptic axon terminal (with synaptic vesicles and mitochondria) forming an asymmetrical synapse with a dendrite (seen in cross sectional profile). An astrocyte partially envelops the synapse. Each component of the central synapse has a complex ensemble of proteins integral to their function. Although the lists are incomplete, some important proteins are identified.



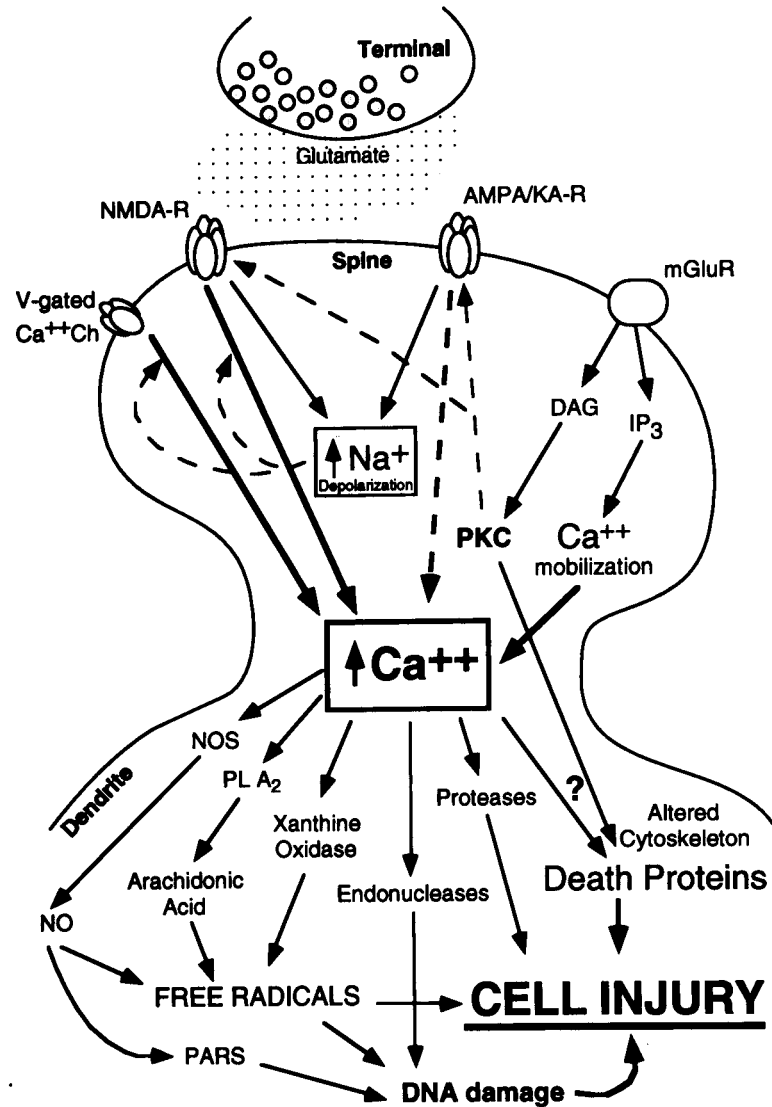


Fig. 2. Summary diagram of synaptic mechanisms related to excitotoxic neuronal degeneration. The diagram summarizes the prominent postsynaptic intracellular pathways that lead to neuronal injury and death resulting from excitotoxic activation of GluR. Abbreviations: AMPA/KA-R, α -amino-3-hydroxy-5-methyl-4-isoxazole propionate and kainate receptors; DAG, diacylglycerol; IP₃, inositol trisphosphate; mGluR, metabotropic glutamate receptor; NMDA-R, *N*-methyl-*D*-aspartate receptor; NO, nitric oxide, NOS, NO synthase, PARS, poly (ADP-ribose) synthetase also known as PARP, poly(ADP-ribose) polymerase; PKC protein kinase C; PLA₂, phospholipase A₂; V-gated Ca⁺⁺ ch, voltage-gated Ca⁺⁺ channel.

electron-dense meshwork of fine filaments that underlies the plasma membrane and is thought to anchor and cluster neurotransmitter receptors (16–18).

Astroglia have a critical role in synaptic function and neuronal survival. Astroglia are the most numerous cellular element of the mammalian CNS. They outnumber

neurons by ten to one and comprise up to 50% of the total tissue volume in some brain regions, with the volume of the astroglial compartment increasing with phylogenetic development (19,20). Astroglial processes ensheath the cell bodies and processes of neurons, envelop synapses (Fig. 1), invest the nodes of Ranvier of myelinated axons, and form glial limiting membranes over the meninges and parenchymal vasculature. The known functions of astrocytes are numerous, including regulation of synaptic neurotransmission, extracellular pH, ion concentration and osmolarity, production and secretion of extracellular matrix proteins, neurotrophic factors and cytokines, detoxification of toxic metabolites (e.g., ammonia, glutamate, and free radicals), and immune/inflammatory response mechanisms (19,20). Astrocytes express functional ligand-gated and metabotropic receptors to neurotransmitters, including glutamate receptors, and they express voltage-gated and other ion channels, including Ca^{2+} , Na^+ , K^+ , and Cl^- channels. Astroglia also possess uptake systems for neurotransmitters for terminating synaptic neurotransmission. Astrocytes respond to a wide variety of potentially injurious stimuli in an effort to restore normal physiological set points. Perturbations in astroglial functions may participate directly in the pathobiology of neuronal degeneration in disease. Important topics that will be discussed in this chapter are astroglial uptake of glutamate and production of amyloid β protein.

2.2. Glutamate Receptor Subtypes

The amino acid glutamate is the major excitatory neurotransmitter in the CNS (21,22). In normal circumstances, glutamatergic synaptic transmission occurs by regulated release of glutamate from presynaptic axon terminals (Figs. 1 and 2). Concentrations of neurotransmitter glutamate at the synaptic cleft have been estimated to be ~ 1 mM, whereas the concentration of interstitial glutamate is ~ 1 μM (23). At synapses, glutamate binds and activates several molecular subtypes of glutamate receptors (GluRs) located on the plasma membranes of neurons and some glial cells (24). These receptors are categorized as members of one of two families, the ionotropic receptors and metabotropic receptors, which differ structurally (distinct molecular compositions) and functionally (distinct signal transduction mechanisms) (24,25).

Ionotropic GluRs form monovalent cation (Na^+ , K^+)-conducting channels, but the different subtypes have differences in their permeabilities to divalent cations (Ca^{2+}) (24,25). The activation of ionotropic GluRs directly changes conductance of specific ions through the receptor-ion channel complex, thereby inducing membrane depolarization. The ionotropic GluRs are the *N*-methyl-*D*-aspartate (NMDA) receptors and the non-NMDA receptors (α -amino-3-hydroxy-5-methyl-4-isoxazole propionate [AMPA] and kainate receptors). These receptors are oligomers, most likely pentameric heterooligomers, of homologous subunits encoded by distinct genes. The NMDA receptor subunits are NR1 and NR2A-NR2D, the AMPA receptor subunits are GluR1-GluR4 (or GluRA-GluRD), and the kainate receptor subunits are GluR5-GluR7 and KA1-2 (24,25). Variants of AMPA receptor subunits (designated as “flip” and “flop”) are generated by alternate splicing of GluR1-GluR4 mRNA (26). These two versions of subunits are different minimally in a 38-amino acid transmembrane sequence, but the “flip” variant shows less desensitization and larger currents (26). Additional diversity in GluR function is generated by RNA editing (25). GluR2 is a

negative regulator of Ca^{2+} permeability through non-NMDA receptors (i.e., its presence in the pentameric receptor assembly blocks Ca^{2+} flux) (25,27–29). The molecular mechanism for the Ca^{2+} impermeability of GluR2 is RNA editing. The genomic sequence of GluR2 differs from the cDNA in the coding sequence between transmembrane domains 1 and 2, and this editing of mRNA transcripts results in an arginine instead of a glutamine, thereby changing the channel properties (27–29).

In most neurons in the CNS, activation of glutamate-gated ion channels produces fast, short-lived, excitatory postsynaptic currents with latency periods ranging from 1–10 msec; excitatory postsynaptic currents of non-NMDA receptors have greater amplitudes and shorter durations than NMDA receptors. AMPA and kainate receptor channels are voltage-independent, whereas the NMDA receptor is voltage-dependent because of a Mg^{2+} block at resting membrane potentials (24,25). Because the non-NMDA receptor channels are permeable to monovalent cations, Na^+ enters the cell following its electrochemical gradient, thereby depolarizing the postsynaptic plasma membrane and activating voltage-sensitive ion channels, including NMDA receptors, Ca^{2+} channels, and Na^+ channels (Fig. 2).

Other GluRs do not form ion channels but are instead linked to signal transduction molecules within the plasma membrane. These metabotropic GluRs (mGluRs) are G protein-coupled receptors that are single proteins encoded by single genes (30,31). mGluRs have slower electrophysiological characteristics (latencies >100 msec) than ion channel GluRs. Group I mGluRs (mGluR1 and mGluR5) operate through activation of phospholipase C (PLC) by G_q proteins, phosphoinositide hydrolysis and generation of inositol-1,4,5 triphosphate and diacylglycerol, and subsequent mobilization of Ca^{2+} from nonmitochondrial intracellular stores (31). Group II mGluRs (mGluR 2 and 3) and Group III mGluRs (mGluR 4 and 6–8) function by G_i or G_o protein-mediated inhibition of adenylyl cyclase and modulation of ion channel activity (30,31).

2.3. Glutamate Transporter Subtypes

Excitatory synaptic transmission is largely terminated by high-affinity, Na^+ -dependent transport of glutamate into cells (neurons and astroglia), thereby preventing extracellular concentrations of synaptic glutamate from reaching neurotoxic levels (Fig. 1B). Five distinct high affinity, Na^+ -dependent glutamate transporters have been cloned from animal and human tissue (GLAST, GLT1, EAAC1, EAAT4, and EAAT5), and these proteins differ in structure, pharmacological properties, and tissue distribution (32–34). Under physiological conditions in normal adult CNS, immunohistochemical studies have shown that GLAST and GLT1 are expressed primarily in astrocytes, whereas EAAC1 is widely distributed in neurons (35–38). EAAT4 is expressed mainly in cerebellar Purkinje cells (37,39), and EAAT5 is primarily a retinal glutamate transporter (34). Thus, the dominant astroglial and neuronal glutamate transporters in cerebellum are GLAST and EAAT4, respectively, whereas GLT1 and EAAC1 are the primary astroglial and neuronal glutamate transporters, respectively, in forebrain, brainstem, and spinal cord.

The different molecular subtypes of glutamate transporters have very distinct, but coordinated, regional patterns of expression during CNS development. The cell-type specificity in the expression of distinct glutamate transporters subtypes is generally similar in the developing and adult CNS. However, one major exception to this conclu-

sion is that GLT1 is also expressed transiently in a variety of neurons in the developing CNS (37,38,40).

2.4. Synaptic Pathobiology: GluR-Mediated Excitotoxicity and Glutamate Toxicity

Abnormalities in synaptic function (Fig. 2) can kill neurons in animal models and in humans. Although glutamate and GluR activation are critical for normal brain function, glutamate is toxic to neurons at abnormally high concentrations (41,42). When GluRs are activated excessively, a process called excitotoxicity ensues (41,42). Acute excitotoxicity causes degeneration in cultures of neurons from animal brain and spinal cord and after *in vivo* delivery of GluR agonists into the CNS of experimental animals (42–45). In addition, excitotoxicity participates in the mechanisms for neuronal degeneration in animal models of cerebral ischemia and traumatic brain injury (11,42,43) and in the neurotoxicity of humans resulting from ingestion of mussels contaminated with the GluR agonist domoic acid (46). In humans, GluR-mediated excitotoxic mechanisms have also been suspected to be responsible for epilepsy, for the neuronal death in the brain resulting from cardiac arrest and stroke, and for neuronal degeneration occurring in individuals with AD, ALS, Huntington's disease, and Parkinson's disease (11,14,42,43,47).

The excessive interaction of glutamate or chemical analogs of glutamate with subtypes of GluRs produces abnormalities in intracellular ionic concentrations, pH, protein phosphorylation, molecular stability, and energy metabolism (42,43). Excitotoxicity challenged cells (Fig. 2) undergo rapid osmotic perturbations and swell, reflecting the influx of Na⁺, Cl⁻, and H₂O (43). An increase in cytosolic free Ca²⁺, resulting from activation of Ca²⁺-permeable AMPA receptors and voltage-sensitive ion channels including NMDA receptors and Ca²⁺ channels, causes increased enzymatic activity of Ca²⁺-sensitive proteases, protein kinases, and phosphatases, endonucleases, and phospholipases (Fig. 2). Through the activation of proteins with DNase activity, excitotoxicity results in internucleosomal DNA fragmentation in cultures of cortical neurons (48,49) and cerebellar granule cells (50,51), although others have not found internucleosomal DNA fragmentation in cerebellar granule cell cultures (52). *In vivo*, internucleosomal and random fragmentation of genomic DNA occur 12–48 h after intracerebral injections of excitotoxins (44,45,53,54), and this pattern can persist to 5 d postlesion (54). The activation of other Ca²⁺-dependent enzyme systems, such as phospholipase A₂, cyclo-oxygenase, and xanthine oxidase, can generate highly toxic reactive oxygen species (ROS) (55,56). Another pathway for the production of ROS, namely nitric oxide (NO), is activation of Ca²⁺/calmodulin-dependent NOS (57).

Oxidative stress is a potent stimulus for neuronal death in cultured neurons (58,59). ROS (e.g., superoxide anion radical, hydrogen peroxide, hydroxyl radical, NO, and peroxynitrite) can cause direct oxidative damage to macromolecules, including DNA, protein, and lipid membranes (55–57). The balance between the formation of endogenous free radicals and antioxidant defense mechanisms is important for cellular survival. ROS are also products of oxidative metabolism. The mitochondrial electron-transfer chain is a primary generator of superoxide and peroxide, and damaged mitochondria are believed to produce greater amounts of superoxide ion (60). Damage to mitochondrial DNA caused by ROS may lead to protein conformational

changes usually associated with an inefficient electron transfer to cytochrome c oxidase (61) and, hence, enhanced superoxide and peroxide formation. Other critical molecular targets for oxidative damage include glutamate transporters (62), Na⁺/K⁺ ATPase (63), glucose transporters (64), cytoskeletal proteins (65,66), and superoxide dismutase-1 (67). ROS can also mediate trophic factor deprivation-induced apoptosis of sympathetic neurons in vitro (68), and we have implicated ROS in target deprivation-induced apoptosis of brain and spinal cord neurons in vivo (69,70). Because neuronal survival depends on trophic factors (71,72), some of which are glial-derived, abnormalities in neurotrophic support may result in apoptotic death of neurons by inducing a PCD mechanism involving the generation of ROS (68–70).

Another mechanism through which glutamate can cause death of neuronal cells is oxidative glutamate toxicity. In this process, glutamate alters cellular metabolism by interacting with the cystine-glutamate antiporter, resulting in depletion of intracellular cystine/cysteine and reduced levels of the cysteine-containing tripeptide glutathione (73). Oxidative stress results from depletion of intracellular pools of the antioxidant glutathione (73). Activation of phosphoinositide-liked mGluRs generates a cellular response that protects against oxidative glutamate toxicity; in contrast, group I mGluR antagonists potentiate glutamate toxicity (74).

3. NEUROGLIAL AND SYNAPTIC MECHANISMS FOR NEURONAL DEGENERATION AFTER CEREBRAL ISCHEMIA

3.1. *Abnormalities in Glutamate Transport and Astroglia Occur after Global Cerebral Ischemia*

Numerous overlapping mechanisms for neuronal injury are likely to be operative in ischemic brain damage. Here, the focus will be on impaired glutamate transport, excitotoxicity, and the associated perturbations in intracellular signal transduction. In models of transient global cerebral ischemia, extracellular glutamate levels are increased transiently (75–78), possibly resulting in excitotoxic activation of neuronal GluR (79). Abnormally high concentrations of extracellular glutamate may be the result of vesicular exocytosis of glutamate (80), reversed glutamate transporter function (81), defective uptake of glutamate (82–84), or astrocyte swelling (85). Of these possible mechanisms, impaired glutamate transporter function has received considerable attention.

Impaired glutamate transport is thought to cause neurodegeneration. For example, intraatrial delivery of the glutamate transport inhibitor DL-threo-3-hydroxyaspartate causes neuronal degeneration in rat (86). Lethal spontaneous seizures and increased susceptibility to acute cortical injury occur in some mice deficient in GLT1 (87), and GLAST gene-ablation exacerbates retinal damage after ischemia in mice (88). However, we have shown that transient reductions in glutamate transporter function and astroglial glutamate transporter (GLAST and GLT1) protein expression can occur in the absence of neuronal degeneration in models of axotomy and deafferentation (89,90).

Alterations occur in glutamate transporters after cerebral ischemia. In adult rat, D-[³H]aspartate binding sites are increased in hippocampus within 5 min after forebrain ischemia (91), but the levels of GLT1 mRNA and protein are reduced in hippocampus at 3–6 h postischemia (92). In rat pups (7-d-of-age), high-affinity glutamate transport is

transiently reduced in striatum during hypoxia-ischemia and after one hour of recovery (82). In hypoxic-ischemic piglets (1-wk-old), high-affinity glutamate transport in striatal synaptosomes is defective functionally by 6 to 12 h recovery (84), and GLT1 and EAAC1 protein levels are reduced in striatum at 24 h recovery and thereafter (83). In contrast, GLAST protein expression is maintained in striatum after hypoxic-ischemic injury (84). Therefore, the evolution of neuronal degeneration in some brain regions after hypoxia-ischemia is paralleled by sustained abnormalities in glutamate transport during the first 24 h of recovery. These defects in glutamate transporters are molecular subtype specific (83,84).

A functional defect in glutamate uptake could be mediated by inactivation of glutamate transporters. For example, covalent modification of glutamate transporters by glycosylation influences their function. *N*-linked glycosylation of EAAC1 (93), but not *N*-glycosylation of GLAST (94), appears to be required for transporter activity. We have shown that the Golgi apparatus within striatal neurons undergoes fragmentation and vesiculation by 3–6 h after hypoxia-ischemia. Furthermore, during this time course, tubulin undergoes extensive oxidative damage (i.e., protein nitration) through a NO-associated toxic pathway. Therefore, posttranslational processing as well as targeting and transport of proteins may be abnormal early after hypoxia-ischemia (11,95).

Covalent modification of glutamate transporters by phosphorylation has subtype-specific modulatory effects on activity. Protein kinase C (PKC) activation results in direct phosphorylation of GLAST and inhibits GLAST function (96); in contrast, phosphorylation of GLT1 by PKC stimulates GLT1 function (97). We have found increases in PKC activity as well as increases in membrane protein phosphorylation at serine sites in vulnerable brain regions at 24 h after global ischemia (98). Thus, phosphorylation of astroglial GLAST, but not GLT1, by PKC may be a mechanism for reduced glutamate uptake in vulnerable brain regions after hypoxia-ischemia (84).

Another possibility is that glutamate transporter function may be impaired by direct structural damage to the proteins. In cell culture systems, oxygen radicals decrease high-affinity glutamate transport (99). Peroxynitrite (formed by the combination of superoxide and NO) is a potent inhibitor of GLAST, GLT1, and EAAC1 function (62,100). We have found evidence for peroxynitrite-mediated oxidative damage to membrane proteins within the piglet striatum early (3 to 6 h) after hypoxia-ischemia (95). Some of these proteins have not yet been identified. It is interesting that the molecular mass of some of these proteins is in the range of glutamate transporters (i.e., ~65–73 kDa). These abnormalities in protein nitration and in the expression or function of glutamate transporters may participate in striatal neurotoxicity after hypoxia-ischemia.

Abnormal control of extracellular glutamate concentrations by astroglia could be an early mechanism for neuronal degeneration after ischemia. In our piglet model of hypoxia-ischemia, striatal astrocytes are damaged early, as evidenced by their swelling, fragmentation, and death as well as by the loss of GLT1 protein at 24 h recovery (83). Astroglia in culture survive exposures to excitotoxins at concentrations sufficient to kill most neurons, suggesting that astrocytes are more resistant than neurons to excitotoxicity; however, overactivation of AMPA receptors is lethal to astrocytes, when receptor desensitization is blocked (101). In addition, astrocytes *in vivo* are damaged by acidosis (102), and prolonged, severe acidosis is lethal to astrocytes *in vitro* (103).

Astroglial swelling occurs within minutes of hypoxia-ischemia, suggesting that initial astrocytic abnormalities may contribute to the evolution of neuronal damage (104). In piglets, principal striatal neurons and astrocytes have a similar time course of injury and death at one to two days following hypoxia-ischemia (83,84,95). Cell culture studies show that neurons in an astrocyte-poor environment are more vulnerable to excitotoxicity than neurons cultured with astrocytes (105) and that uptake of glutamate by astrocytes improves neuronal survival in an excitotoxic environment (106). The finding that astroglial and neuronal injury occur concurrently within brain further strengthens the concept that abnormalities in astroglia could participate in the evolution of neuronal degeneration after hypoxia-ischemia (83,104). Astroglial damage and defective glutamate transport would favor excitotoxic neuronal cell death after hypoxia-ischemia, and oxidative damage to astroglial proteins could be a potential molecular mechanism for this defect (83,84,95).

3.2. Glutamate Receptor-Mediated Excitotoxicity as a Mechanism for Neuronal Cell Death After Cerebral Ischemia

Neuronal cell death after ischemia involves perturbations in intracellular Ca^{2+} homeostasis and impairments in protein synthesis. The GluRs that modulate intracellular Ca^{2+} levels within neurons are the ion channel receptors (NMDA and AMPA) and the group I mGluRs (24–31). In models of transient global cerebral ischemia a possible role for excessive activation of GluRs in the mechanisms for delayed neuronal death is supported by studies showing that blockade of AMPA receptors is neuroprotective following forebrain ischemia in adult rodents (107) and that abnormalities in phosphoinositide signaling pathways occur in hippocampus after ischemia (108). In models of focal ischemia (stroke), antisense oligodeoxynucleotide knockdown of NR1 production (109), targeted disruption of the NR2A gene (110), and selective pharmacological antagonism of NR2B (111) attenuate forebrain damage, thus a decrease in the number of functional NMDA receptors appears to mediate this neuroprotection.

We have demonstrated that excitotoxic activation of GluRs causes an apoptosis-necrosis continuum for cell death in which the structure of neuronal degeneration is influenced by the subtype of GluR that is activated and the age (maturity) of the CNS at the time of the insult (44,45). In the developing rat CNS, excitotoxic activation of NMDA and non-NMDA GluRs causes neuronal death with phenotypes ranging from apoptosis to necrosis. Excitotoxic neuronal death occurs as three structurally different forms: Classic apoptosis, classic necrosis, and a hybrid of apoptosis and necrosis (11,44,15,47). In contrast, in the adult rat CNS, the degeneration of neurons caused by NMDA receptor activation is necrosis morphologically; however, the neuronal death produced by non-NMDA receptor activation appears to be a structural hybrid of apoptosis and necrosis and is distinct from the death caused by NMDA receptor stimulation.

Interestingly, neuronal death after cerebral ischemia may not follow the apoptosis-necrosis continuum for excitotoxic neuronal death. We have shown with three different animal models of transient global ischemia that neuronal cell death within selectively vulnerable brain regions (e.g., CA1 pyramidal neurons, cerebellar Purkinje cells, and striatal neurons) is identical structurally to excitotoxic neuronal cell necrosis mediated by NMDA receptor activation, and, in the vulnerable neuronal populations, classic apoptosis and hybrids of apoptosis-necrosis rarely occur (11,47,112). However, in sev-

eral different paradigms of global ischemia, we and others have been unable to rescue these neurons from degeneration using noncompetitive antagonists to NMDA receptors (113,114) or competitive antagonists to NMDA receptors (115,116), AMPA receptors (117–119), and group I mGluRs (120). Some of our data even suggest that antagonists to NMDA receptors (116), AMPA receptors (118,119) and mGluRs (120) worsen neurologic outcome after ischemia. Yet, other studies have shown that AMPA receptor antagonism is neuroprotective in adult rodent models of global ischemia (107,114). The lack of neuroprotection and the deleterious effects of GluR antagonists in *in vivo* animal models are difficult to interpret and may be related to several variables associated with experimental design, including the dosage, solubility, delivery, administration route, and systemic pharmacokinetics of the drug. Although GluR-mediated excitotoxicity and glutamate toxicity continue to be potential mechanisms for neurodegeneration after hypoxia-ischemia, the pathobiology of this neuronal cell death remains poorly understood. The future clarification of the structural and molecular mechanisms of neuronal death after ischemia and the neuroglial participation in these mechanisms will lead to a rational and effective design of experiments for the protection of neurons and glia.

3.3. Possible Roles for Protein Kinases in Neurodegeneration After Ischemia

Protein kinases function in cell growth and differentiation, and, in nervous tissue, additional functions are regulation of neurotransmitter receptors and ion channels as well as modulation of neuronal excitability, neurotransmitter release, and synaptic plasticity (Fig. 2; for review *see* refs. 121,122). Protein kinase activation may thus play a role in GluR-mediated excitotoxic neuronal death (123,124) and in neuronal degeneration after ischemia (98,122,125). GluR stimulation causes increased intracellular Ca^{2+} and activation of Ca^{2+} /calmodulin protein kinase II (CaM kinase II) and causes increased activity of phospholipase A_2 and PLC (Fig. 2), leading to the accumulation of diacylglycerol and activation of PKC (24,31,43). Mild ischemia induces a persistent translocation of CaM kinase II from the cytosol to synaptic plasma membranes (126,127). We have identified a sustained increase in PKC activity, an isoform-specific translocation to the plasma membrane, as well as increased protein phosphorylation at serine residues in plasma membranes of selectively vulnerable brain regions after ischemia (98). Astroglia may contribute to some of these changes (98). Other studies have suggested that protein kinases that are membrane-translocated after ischemia function improperly (126,128). However, changes in brain protein kinase activity may depend on the severity of ischemia and energy failure (98).

Several studies have shown that phosphorylation of AMPA, kainate, and NMDA receptors by CAM kinase II, PKC, or cAMP-dependent protein kinase A causes positive modulation of receptor function by potentiating activation (129–134). This enhancement of receptor function is transient because of the activity of postsynaptic protein phosphatases (134). In contrast, PKC-mediated phosphorylation of PLC-coupled mGluRs causes desensitization to glutamate (135,136). The integrative actions of different molecular subtypes of GluRs are also being revealed. Activation of PLC-coupled mGluRs increases NMDA receptor function through PKC activation (137,138). PKC potentiates NMDA receptor function by increasing the probability of channel openings and by reducing the voltage-dependent Mg^{2+} block (139).

The possible relevance of these observations to neuronal and astroglial degeneration after ischemia is provocative but still only speculative. We have found increases in PLC and PKC after ischemia (98,140), and other groups have found abnormalities in phosphoinositide signaling pathways after ischemia (108). Electrophysiological studies of neuronal activity in vulnerable regions after transient ischemia are discrepant. By extracellular recording in vivo, studies have shown that spontaneous firing rate in CA1 is increased (141), whereas other studies show that spontaneous firing rate and evoked responses are suppressed (142,143). By intracellular recording, CA1 neuronal activities and evoked synaptic potentials are suppressed after in vitro hypoxia-ischemia (144); similarly, in spiny striatal neurons in vivo, spontaneous activities and evoked postsynaptic potentials are suppressed and excitability is decreased after transient ischemia (145). However, whole-cell recordings reveal that CA1 pyramidal neurons have abnormal excitatory postsynaptic currents produced by activation of non-NMDA receptors that mediate Ca^{2+} influx (146). It is still uncertain whether changes in protein kinases after ischemia mediate these functional abnormalities and contribute directly to the mechanisms of neuronal death or whether they reflect secondary degenerative or regenerative processes in injured or surviving neurons and/or changes due to activation of glial cells. Based on the knowledge that protein kinases modulate GluR receptor and glutamate transporter function, ion channel function, Na^+/K^+ ATPase activity, and neurotransmitter release (to name only a few critical functions for protein kinases), much more work is needed to identify the possible contributions of protein kinases to the mechanisms for neuronal degeneration after ischemia (Fig. 2).

3.4. Participation of NO-Mediated Mechanisms in the Pathobiology of Neurodegeneration After Ischemia

NO is a short-lived, membrane-permeant ROS that functions in synaptic neurotransmission, synaptic plasticity, cell-mediated immune response, and cerebrovascular regulation (57,147,148). NO is generated by the catalytic activity of NOS, of which three molecular isoforms exist. Brain or neuronal NOS (NOS1) is constitutively expressed in subsets of neurons, inducible NOS (NOS2) is an inflammatory/glial cell isoform, and endothelial NOS (NOS3) is constitutively expressed in vascular endothelial cells (57,148–150). Neuronal NOS activity is regulated by intracellular Ca^{2+} (149). NMDA receptor activation results in NOS activation (Fig. 2; 151), and mGluR activation enhances NO production, possibly involving a Ca^{2+} -induced Ca^{2+} release amplification of mGluR-associated intracellular signals (152). NO has presynaptic actions as well by stimulation of synaptic vesicle exocytosis in a process involving cGMP (153). NO functions by forming covalent linkages and redox interactions with intracellular proteins (154). Excessive generation of NO is thought to be toxic to cells in vivo and may be an important mediator of neuronal degeneration resulting from cerebral ischemia (57,155–157), CNS inflammation (158), and axotomy/target deprivation (70). A predominant mechanism by which NO toxicity occurs is by the diffusion-limited reaction of NO with superoxide to generate peroxynitrite anion, which is a strong and relatively long-lived oxidant that directly damages the structure of a variety of proteins (57). Peroxynitrite decomposes to form secondary oxidants, including the particularly toxic hydroxyl radical (57), which we have shown to damage neuronal DNA and RNA after cerebral ischemia (95,159) and axonal injury (69,70).

In studies of cerebral ischemia, the results supporting a role for neurotoxic actions of NO *in vivo* are discrepant. In settings of focal ischemia, mice deficient in neuronal NOS (155) or inducible NOS (156) have slightly smaller infarct volumes than controls, and pharmacological inhibition of NOS has been shown to decrease brain damage volume in some studies (160) but not in other experiments (161,162). In settings of global ischemia, mice with targeted disruption of the neuronal NOS gene have less CA1 damage than wild-type mice (163). However, in rat and gerbil, pharmacological inhibition of NOS is not protective (162,164). We have been unable to show that an inhibitor of NOS results in neuroprotection in hippocampus and cerebellum after global ischemia (165). However, we have found that peroxynitrite-mediated damage to striatal membrane proteins occurs strongly by 6 h after hypoxic-asphyxic cardiac arrest (95,159). In addition, we discovered that the critical cytoskeletal protein tubulin is a target of peroxynitrite (159). It is therefore possible that the participation of NO-mediated toxicity is different in acute vs delayed neuronal degeneration after cerebral ischemia, and, thus, onset and severity of oxidative stress may influence rate of neuronal death on a regional basis, because striatal damage evolves faster than CA1 neuron and Purkinje cell damage (11).

4. SYNAPTIC PATHOBIOLOGY IN PROGRESSIVE, AGE-RELATED NEURODEGENERATIVE DISEASES

4.1. Synaptic Dysfunction in AD

AD is the most common type of dementia occurring in middle and late life (166), and it affects 7–10% of individuals >65 years of age and possibly 40% of people >80 years of age (12,167). The prevalence of AD is increasing proportionally to increased life expectancy (estimates predict that ~25% of the population will be >65 years of age in the year 2050). AD now affects >4 million people in the United States (168). Although most cases of AD have unknown etiologies and are called sporadic, some cases of AD, particularly those with early onset, are familial and are inherited as autosomal dominant disorders linked to mutations in the gene that encodes amyloid precursor protein (169–171) or genes that encode for proteins called presenilins (172,173). For late onset sporadic cases, a variety of risk factors have been identified in addition to age. The apolipoprotein E (apoE) allele is a susceptibility locus with the apoE4 type showing dose-dependent contributions (174). Cardiovascular disease and head trauma are additional risk factors for AD (166).

The mechanisms that cause the profound neuronal degeneration and the progressive impairments in memory and intellect that occur with sporadic and familial AD are not understood. Neuronal survival and normal memory and cognition depend on synaptic function. Regulated exocytosis of neurotransmitter-containing vesicles (Fig. 1A) is obligate for normal synaptic function (15). Synaptophysin (p38), an integral membrane glycoprotein of small synaptic vesicles in the presynaptic terminal (15), is one protein of many that functions in regulated exocytosis (Fig. 1B). We have found that synaptophysin is reduced in the hippocampus of individuals with AD who have moderate to severe deficits in memory (5). This finding is not surprising in light of the vulnerability of the hippocampus in advanced AD (1,3,4,166). A more exciting finding is the loss of synaptic marker in individuals with early AD (or possible AD)

who had no detectable cognitive impairment (5). The severity of this abnormality in synaptophysin in hippocampus correlates strongly with the severity of memory impairment in individuals with AD. Defects in other presynaptic components for regulated exocytosis of neurotransmitters (Fig. 1B) may also occur in the hippocampus of individuals with early AD. We also have interesting new data suggesting that synaptobrevin, synaptotagmin, syntaxin, and Rab3a are abnormal in the hippocampus of AD, but synapsin I, SNAP25, and SV2 levels are normal (175). Thus, presynaptic vesicles appear to be more vulnerable in AD than the presynaptic membrane that contains the active zone where vesicle docking occurs. In addition, we have discovered in individuals with AD selective abnormalities in the levels of the NR2B subunit of the NMDA receptor in the hippocampus as well as abnormalities in NMDA receptor phosphorylation (176). These synaptic molecule defects in subjects with early AD may foreshadow the clinical appearance of memory/cognitive impairment. Ultimately, structural loss of the entire synaptic complex could contribute to the atrophy of the cerebral cortex (i.e., cortical volume loss) which correlates strongly with cognitive decline (177).

4.2. Cellular Dynamics of Senile Plaque Formation and Amyloid Deposition within the Brain During Aging

Senile plaques (SP) are brain lesions that occur in individuals with AD, patients with Down's syndrome (DS), and, less frequently, in people aging normally (1,4,178,179, see ref. 2 for additional references). These lesions have a complex composition, consisting of dystrophic neurites (damaged and swollen dendrites or axon terminals), activated astrocytes and microglia, and extracellular deposits of insoluble amyloid fibrils (179, see ref. 2 for additional references). These fibrils are composed of a 4-kDa peptide (A β) (180–182) consisting of 40–42 amino acid residues (183). This protein fragment is derived proteolytically from the amyloid precursor protein (APP), a cell surface protein with a large N-terminal extracellular domain that contains 22 residues of A β , a hydrophobic membrane-spanning region including the transmembrane portion of the A β region, and a short C-terminal cytoplasmic segment (184–186). The APP gene is located on human chromosome 21 (187–189), and mRNA gene products are spliced alternatively to generate ~5 different forms of APP transcripts and protein isoforms (184,189). A role for APP in the pathogenesis of AD is supported further by the identification of mutations in the APP gene linked to early-onset AD in some families (169–171).

The functions of APP are still not well defined, although it appears that APP functions at synapses (2,190). APP is an abundant and ubiquitous protein within CNS and other tissues. However, mice deficient in APP show no major neurological or neuropathological abnormalities (191), possibly due to homologous proteins. APP has structural features similar to some cell surface receptors (184) and may be a G protein-coupled receptor (192). Secreted and nonsecreted forms of APP exist (193,194), with different APP derivatives showing neurotrophic or neurotoxic actions. APP is incorporated into the extracellular matrix (195) and, thus, may have roles in cell-cell and cell-substrate adhesion (193,196–199). Furthermore, APP may function in the regulation of neurite outgrowth (200–201), perhaps by mediating the effects of nerve growth factor (202) and in neuronal and glial responses to brain injury (203–205).

In cell culture, APP normally undergoes constitutive proteolytic cleavage (186,190,193) by an α -secretase, an enzyme that cleaves APP within the A β region at or near the plasma membrane (206–208), thereby generating secreted forms of APP and precluding the formation of full-length A β peptide fragments (206,208). APP is also metabolized by an endosomal-lysosomal pathway that, unlike the α -secretase pathway, yields amyloidogenic fragments of A β (209,210). A β can be formed normally in vivo and in vitro (211,212), and studies of cultured human cells show that it is generated intracellularly (213,214). Although A β has been shown to be neurotoxic in cell culture, a causal role for A β in neuronal degeneration in vivo remains speculative. A β -secretase cleaves APP at the N-terminus of A β , and a γ -secretase cleaves APP at the C-terminus of A β , causing the formation of A β that is either 40 amino acids or 42 amino acids long (215). This pathway for APP metabolism is found within the endoplasmic reticulum and Golgi apparatus of neurons (216,217). Interestingly, presenilins, which are present at relatively low levels in brain (218), localize to the endoplasmic reticulum and Golgi apparatus (219), and mutant presenilins promote A β 42 generation (220). Mutant presenilin is processed differently than normal presenilin, and fragments that are normally subject to endoproteolytic cleavage tend to accumulate (221). Thus, metabolism of APP through the β - and γ -secretase pathways may be promoted by presenilin-1 and presenilin-2 gene mutations linked to early onset familial AD.

We and others have shown that APP is expressed by neurons and by subsets of astroglia, microglia, and vascular endothelial cells (2,196,222–224). The most prominent neuronal localization of APP is within cell bodies and dendrites and is particularly enriched postsynaptically at subsets of synapses (2,205). The expression of APP in nonneuronal cells in brain is low in comparison to the dominant expression of APP within neurons and their processes. It appears that astroglia and microglia constitutively express APP at low levels in the resting state (2,222). However, an important finding is that the relative enrichment of APP within these neuroglial cells changes in response to brain injury and synaptic abnormalities (2,204). This idea is supported by our finding that APP is expressed prominently by activated astroglia and microglia within SP of aged nonhuman primates (2,224) and by other reports (225) showing that APP is localized to astrocytes in SP in cases of AD. Other studies have shown that APP isoforms containing the Kunitz protease inhibitor domain are expressed in reactive astrocytes in early stages of brain damage (204). Because levels of APP in some neurons and nonneuronal cells are increased by the cytokine interleukin-1 (226), it is likely that the expression of APP is inducible in glia when these cells are activated in response to neuronal injury.

Several hypotheses for A β deposition and SP formation have been presented. The genesis of SP may begin with the formation of extracellular A β before the degeneration of cellular elements within these lesions (227,228). Alternatively, A β may be derived from degenerating axonal nerve terminals or dendrites containing APP that evolve into neurite-rich foci that form A β at the cell plasma membrane by aberrant processing of APP within neurons (181,223,229). In addition, invading reactive microglia (2,179,230,231) and astroglia (2) as well as capillaries (232) may actively produce A β from APP. In one scheme for SP formation, A β (in a nonfibrillar form and then a fibrillar form) is deposited before neuronal neuritic damage occurs, and thus is a cause of neuronal degeneration (233,234). We have not identified extracellular A β (even as

sparse, scattered bundles of fibrils) before the appearance of synaptic abnormalities and dendritic/axonal neurites (2,224). We believe that astroglia and microglia are primary generators of A β deposits in the aging brain (2,224). In early diffuse plaques, we have identified A β -containing transformed astrocytes and microglia after neuritic defects have appeared. Our experiments with aging nonhuman primates are concordant with studies of SP in the cerebral cortex from cases of AD (179,230).

These studies reveal that SP are dynamic brain lesions that evolve from early synaptic defects within the neuropil to mature plaques and extracellular deposits of A β . The staging of these lesions is thought to be the degeneration of neuritic structures, followed by the attraction of reactive glia, and the subsequent deposition of extracellular A β derived from microglia (2,179,230) or astrocytes (2). Our studies demonstrate that structural and biochemical perturbations within neuronal and nonneuronal cells occur before the deposition of extracellular A β fibrils (2,224). Furthermore, our results suggest that focal abnormalities in synaptic contacts within the neuropil (synaptic disjunction) may initiate this complex series of events resulting in the formation of diffuse SP and deposits of A β . In response to synaptic disjunction in the aged brain, astroglia and microglia produce A β (2). The molecular pathology that we and others have identified at the synaptic level in humans with AD (5,175,176,235) may be related to SP lesions and A β deposits. For example, we found a strong inverse correlation between synaptophysin loss and the density of neuritic and diffuse plaques in hippocampus. It is not surprising to find an inverse correlation between SP density and synaptophysin immunoreactivity, because synaptic disjunction in the neuropil and abnormal APP processing within neuroglia may be early events in the formation of SP (2,224).

4.3. Oxidative Stress in the AD Brain

Neurons have a particularly high risk for structural damage caused by ROS, because of high rates of oxygen consumption and high contents of polyunsaturated fatty acids that are susceptible to lipid peroxidation; therefore, a free radical hypothesis has been suggested as a mechanism for compromised CNS functioning with aging (3,11,47,66). Some experimental evidence is available to support this argument. For example, superoxide dismutase-1 (SOD1) activity is elevated in fibroblasts from individuals with familial AD (236), and the SOD1 gene is located on human chromosome 21 (237), which individuals with DS possess an extra copy, and a gene dosage effect on lipid peroxidation has been observed in brain tissue of patients with DS (238). ROS have been implicated in the induction of neuronal death by apoptosis in DS (239) and in *in vitro* (58,59) and *in vivo* models (11,69,70). Mitochondria may participate in the effector stage of apoptosis by directly providing a rich source of ROS or by changes in mitochondrial membrane cell death proteins that prevent apoptosis by an antioxidant mechanism, or that suppress apoptosis by blocking release of cytochrome c (a required molecule for apoptosis in cell culture systems) or by regulating membrane potential and volume homeostasis of mitochondria (for references see 10,11,69,71).

Cellular oxidative stress and antioxidant enzyme regulation are relevant to age-related neurodegenerative disorders because abnormalities in antioxidant enzyme systems (e.g., superoxide dismutase) and oxidative injury may provide mechanisms for neuronal degeneration. Cytosolic copper/zinc-superoxide dismutase (SOD1) and mitochondrial manganese-superoxide dismutase (SOD2) are localized predominantly to

neocortical and hippocampal pyramidal neurons, but are scarcely seen in glial cells in normal human brain (3). In AD and DS, the cellular expressions of these two forms of SOD are very different from normal individuals (3). SOD1 is highly enriched in pyramidal neurons undergoing degeneration, whereas SOD2 is more enriched in reactive astrocytes than in neurons. In SP, astrocytes highly enriched in SOD2 surround SOD1-enriched neurites. Some pyramidal neurons coexpress SOD and the cytoskeletal protein tau, and some SOD1-enriched structures in SP are tau-positive (3). Microglia infrequently show expression of high levels of superoxide dismutase. These findings in AD and DS support a role for oxidative stress in neuronal degeneration and SP formation. The differential localizations of SOD1 and SOD2 in sites of degeneration in diseased brain suggest that the responses of cells to oxidative stress is antioxidant enzyme-specific and cell type-specific and that these two forms of superoxide dismutase may have different functions in brain antioxidant mechanisms in response to injury.

A glial expression of SOD has been reported in AD brain (240). Our studies have shown that SOD protein is rarely seen in glial cells in control brain (3). However, in AD and DS brain, SOD2 is induced strongly in reactive astrocytes in cerebral cortex and white matter. This change in the astroglial expression of SOD2 in the brains of AD and DS cases is a major difference between control and diseased tissue, and is possibly due to metabolic activation of reactive astrocytes, because SOD2 is localized within the mitochondrial matrix (241). SOD1-expressing astrocytes are also seen, but this expression is weaker than that of SOD2. Because SOD2 mRNA is induced by tumor necrosis factor- α and interleukin-1, SOD2 is thought to have a cytoprotective function against various microglia/macrophage-derived inflammatory mediators (242,243) that may participate in the pathogenesis of AD and DS (244). Our findings that SOD2 may be downregulated in pyramidal neurons and upregulated in reactive astrocytes in AD and DS brain (3) may relate to the selective cytotoxicity of microglia-derived cytokines (e.g., tumor necrosis factor- α and interleukin-1) (245). Activated microglia secrete superoxide radicals (246), and stimulated neutrophils disrupt SOD structure in cell culture (247), but reactive microglia-macrophage cells that are widely distributed in AD and DS cases, show scarce evidence for expression of SOD (3). Thus, modulation of glial expression of SOD appears to be relatively selective for astrocytes, because in AD and DS, astroglia show the greatest increase in SOD2 expression.

4.4. Synaptic and Astroglial Pathobiology in ALS

ALS is a disease that causes progressive weakness, muscle atrophy, and eventual paralysis. Death occurs within 3 to 5 years of onset and is characterized neuropathologically by progressive degeneration of upper and lower motor neurons in the brain and spinal cord (13,248). The mechanisms leading to the selective degeneration of motor neurons in ALS are not known. Some forms of ALS are inherited (8,9). Mutant forms of SOD1 have been identified in a small subset (10–20%) of individuals with familial ALS which occurs in 5–10% of all patients with ALS (8,9). Forced expression of mutant forms of the gene encoding SOD1 results in degeneration of motor neurons in mice (249,250). A toxic gain in function of mutant SOD1 might initiate this neurodegeneration (249,251). In cultured cells, expression of mutant SOD1 can induce abnormalities in the production of ROS and neuronal apoptosis (251,252). Oxidative stress

resulting from downregulation of normal SOD1 can also cause apoptosis in cell culture (253). Other possible causes for neuronal death in ALS are neurofilament abnormalities (254–256), defective uptake of glutamate by astroglia (6,7) leading to GluR-mediated excitotoxic neuronal death, or deficiencies in neurotrophic factors (257).

We have found (10) that the degeneration of motor neurons in sporadic and familial ALS is different structurally from the motor neuron degeneration found in transgenic mice overexpressing the familial ALS mutant forms of SOD1 (249,250) and in transgenic mice overexpressing normal and mutant neurofilament proteins (254–256). SOD1 and neurofilament transgenic mouse models of ALS have not revealed structural or biochemical changes for apoptotic death of motor neurons similar to those we have discovered in humans with ALS (10). In fact, it is still uncertain whether motor neurons in these mouse models die or whether they remain in a severely atrophic state. The survival of mice with familial ALS mutations is prolonged when crossed with mice overexpressing Bcl-2 (258) or a dominant negative inhibitor of caspase-1 (259), although the degeneration of motor neurons is not prevented (258), suggesting that neuronal degeneration in these mice is not apoptosis controlled by programmed cell death mechanisms. The prominent vacuolar and edematous degeneration of motor neurons in mice overexpressing mutant SOD1 (249,250) or neurofilament protein (254–257) more closely resembles excitotoxic neurodegeneration (45) or transsynaptic neuronal atrophy (but not death) in response to deafferentation (260).

We and others have proposed that excitotoxicity may explain the selective neuronal cell death in ALS (6,7,11,42,47). This hypothesis was formulated based on background information showing that abnormal activation of GluR can kill neurons (41–43) and that exogenous glutamate analogs may be responsible for the damage to upper motor neurons in lathyrism via actions at specific GluRs (42,261). Subsequently, studies revealed that serum and cerebrospinal fluid concentrations of glutamate are increased in some patients with sporadic ALS (262,263), but this change was not found in other studies (264,265). In more recent studies, it has been shown that spinal cord and affected brain regions of patients with sporadic ALS have reduced high-affinity glutamate uptake (6) and a selective reduction in GLT1 (7). It has also been reported that aberrant splicing of astroglial GLT1 mRNA is ALS-specific and is the cause for reduced expression of GLT1 protein and even possibly the cause of motor neuron degeneration (266). However, defective astroglial glutamate uptake and loss of GLT1 occur also in brain regions selectively vulnerable in AD (267), in brain regions vulnerable to hypoxia-ischemia (83,84), and even in brain regions that do not develop neuronal degeneration after axotomy and deafferentation in experimental animals (89,90). In addition, splicing of GLT1 mRNA transcripts is highly variable in human CNS tissue, and variant transcripts considered to be “aberrant” are found in normal individuals and in other species (268–270). Furthermore, the degeneration of motor neurons in individuals with ALS is structurally very different from excitotoxic neuronal degeneration in the adult CNS (10). Thus, it is unlikely that the abnormalities in astroglial GLT1 and GluR-mediated excitotoxicity are the specific causes for motor neuron degeneration in ALS.

Over the years it has become increasingly clear that selective neuronal vulnerability in ALS is not simply related to excitotoxic processes. Neither differential subunit compositions of ion channel GluRs (AMPA and NMDA receptors) nor differential expres-

sions of glutamate transporters appear to explain sufficiently the mechanisms that dictate the selective vulnerability of neurons in motor cortex and motor neurons in brainstem and spinal cord in ALS. This conclusion is based partly on glutamate receptor/transporter expression and cellular localization studies in animal and human CNS performed by us (35,37,38,40,271,273) and by others (36,274,275). The foundation for the excitotoxicity theory in the pathogenesis of ALS is built on experiments showing a selective loss of the astroglial glutamate transporter GLT1 in vulnerable regions in ALS (7). Nevertheless, this observation is still indirect evidence for GluR-mediated excitotoxicity, and the explanations for this abnormality and the disease specificity for motor neurons are controversial. It is also still uncertain whether this abnormality is related to the causes or the consequences of the disease. Most patients with ALS are maintained on mechanical ventilation, as a means of relieving symptoms of chronic hypoventilation and for prolonging life (276), but patients with ALS die eventually from respiratory insufficiency (10,276). In fact, it has been argued that riluzole, a drug that may improve the survival of ALS patients, acts as a Na⁺ channel blocker and increases resistance to hypoxia (by reducing energy demand), not by anti-excitotoxic actions (277). In support of this recent interpretation (277), we have observed in a physiologically well-characterized animal model of cerebral hypoxia-asphyxia that regionally selective abnormalities occur in astroglial GLT1 expression (83,84). Moreover, our new data reveal that non-NMDA and NMDA GluR toxicity in adult CNS neurons is structurally different from the pattern seen in ALS (10,11,45). Thus, it is possible that changes in astroglial glutamate transporters in individuals with ALS are consequences of neurodegeneration, or, alternatively, these changes are related to premortem agonal state.

We have found recently in adult animal models of peripheral nerve avulsion that motor neuron degeneration is induced by oxidative stress and is apoptosis (70). We have also found that motor neuron death in ALS is apoptosis that may be mediated by programmed cell death mechanisms (9,10,282). The neuronal degeneration in ALS closely resembles neuronal apoptosis induced by target deprivation in the mature CNS (10,11,69,70) and could be related to the deficiency in neurotrophic factors that is found in individuals with ALS (257). Some of these neurotrophic factors are produced by neuroglia. Trophic factor deprivation-induced motor neuron apoptosis in cell culture and in developing animals (278–280) is caspase- Bax-, and p53-dependent, as might be the case for motor neuron apoptosis in ALS (10,281,282). Our model of motor neuron apoptosis in the spinal cord will be useful for identifying the synaptic and molecular pathobiology of neuronal apoptosis in the adult CNS and thereby will provide insight into the mechanisms, including the roles for neuroglial-derived trophic factors, for the neuronal degeneration in ALS (281,282).

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Astrocytes and Ammonia in Hepatic Encephalopathy

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1. INTRODUCTION

Hepatic encephalopathy (HE; hepatic coma) refers to a complex neuropsychiatric syndrome resulting from severe liver failure. It is probably the best example of a neurological condition in which astrocytes appear to play a dominant role in its pathogenesis. This disorder gave genesis to the concept of a primary gliopathy, whereby an initial disturbance in astroglial function leads to abnormal neuronal activity (1,2). Studies in HE have additionally provided important insights into the role of astrocytes in ammonia and amino acid neurotransmitter metabolism, and greatly contributed to the evolution of the concept of glial-neuronal interactions/trafficking.

This chapter will review the role of astrocytes in the pathogenesis of HE. Because ammonia is a prime candidate as the neurotoxin in HE, the effects of ammonia on astrocytes will be highlighted with emphasis on its role in glutamatergic and GABAergic neurotransmission and its involvement in astrocyte swelling, a major complication of acute HE. This article also has relevance to clinical conditions associated with hyperammonemia, such as urea cycle disorders and Reyes syndrome.

2. CLINICAL CONSIDERATIONS

HE is often referred to as portal-systemic encephalopathy, which usually occurs in the setting of alcoholic liver cirrhosis. Early manifestations may be quite subtle and only established with formal neuropsychologic tests. As the process progresses patients manifest disturbances in behavior, personality, intellectual capacity, and sleep patterns. They also may display lethargy, hyperventilation, and abnormal motor function (incoordination, ataxia, asterixis). In severe cases there is a depression in the level of consciousness, including coma and death. HE is associated with characteristic electroencephalographic changes (3) and hyperintensities of the globus pallidus on T1-weighted magnetic resonance images (MRI) (4,5).

HE is usually precipitated by gastrointestinal hemorrhage, high protein diet, constipation, infection, excessive diuresis, electrolyte imbalance, or the use of sedatives and hypnotics. With appropriate treatment, chronic HE is usually reversible. However, following repeated bouts of encephalopathy, irreversible anatomical changes occur, especially in the basal ganglia and cerebral cortex (acquired chronic hepatocerebral

degeneration), or in the spinal cord (shunt myelopathy or portal systemic myelopathy). These patients present with dementia, ataxia, dysarthria, intention tremor, choreoathetosis, and spastic paraparesis.

HE may also present in an acute form (fulminant hepatic failure; FHF) that generally occurs following viral hepatitis, drug hepatotoxicity (acetaminophen, halothane, nonsteroidal antiinflammatory agents), or after exposure to various hepatotoxins (mushroom/*Amanita phalloides*, volatile hydrocarbons). Acute HE manifests itself with the abrupt onset of delirium, seizures, and coma. The principal cause of death in acute HE is brain edema associated with increased intracranial pressure.

3. PATHOLOGY

The histopathology of HE in humans and experimental animals is dominated by astroglial changes. Acute HE is characterized by massive astrocyte swelling. This may be accompanied by neuronal necrosis secondary to cerebral ischemia which is mediated by the elevated intracranial pressure.

Alzheimer type II astrocytosis (protoplasmic astrocytosis, metabolic gliosis) is the histological hallmark of chronic HE (3,6). This change is characterized by an apparent increase in the number of astrocytes with enlarged, pale nuclei, peripheral margination of chromatin and often prominent nucleoli. Excessive amounts of lipofuscin granules are occasionally identified. Ultrastructural studies in experimental models of HE have shown an increase in the number of mitochondria, smooth and rough endoplasmic reticulum, and cytoplasmic glycogen, suggesting that astrocytes are metabolically activated. Although unproven, it is generally believed that these glial changes are reversible. Eventually, degenerative changes ensue characterized by cell swelling and the presence of cytoplasmic vacuoles and degenerated mitochondria (7). No significant or consistent neuronal changes have been identified.

Acquired chronic hepatocerebral degeneration is associated with irreversible damage and is characterized by cortical laminar necrosis, microcavitation in the cortex and basal ganglia, and cerebral and cerebellar atrophy, along with degeneration of the white matter columns in the spinal cord. Characteristic features include the presence of Alzheimer type II astrocytes, Alzheimer type I astrocytes (large atypical reactive astrocytes), and nuclear glycogen inclusions (8). These changes are very similar to those seen in Wilson's disease. These abnormalities may be due to an accumulation of manganese (9) (*see Subheading 4.*).

4. PATHOPHYSIOLOGY

The basis for the neurological disorder in liver failure remains elusive. The dominant view over many decades has been that gut-derived nitrogenous products are not detoxified by the diseased liver or are not extracted by the liver as a result of vascular (portal-systemic) shunts that commonly occur in chronic liver disease. These toxins then enter the central nervous system (CNS) and exert deleterious effects. These vascular shunts may occur spontaneously as a consequence of liver disease, or may be iatrogenically induced by the surgical construction of a portacaval anastomosis or a transjugular intrahepatic portosystemic shunt (TIPS) performed to reduce portal hypertension. The presence of a normal liver in these cases highlights the importance of systemic shunting in the pathogenesis of HE.

Various toxins have been invoked over the years, including ammonia, short-chain fatty acids, mercaptans, phenols, biogenic amines, endotoxin, and “middle molecules,” which may act synergistically (10). More recently a role for manganese has been proposed (11). Additionally, abnormalities in GABAergic and glutamatergic neurotransmission have been invoked. These prevailing hypotheses are not necessarily mutually exclusive, as will be developed below. Abnormalities in monoamines and monoaminergic neurotransmission (12), as well as opiate-mediated neurotransmission (13) have also been proposed.

4.1. Ammonia

Of all of the toxins implicated in the pathogenesis of HE, ammonia has received the greatest emphasis for the following reasons:

1. Factors that lead to increased levels of blood or brain ammonia worsen HE (10);
2. Almost all of the effective therapies in HE bring about a reduction in blood ammonia levels (14);
3. Procedures that increase blood or brain ammonia in experimental animals reproduce the clinical and pathological changes of HE (6);
4. Patients with hereditary hyperammonemia have similar clinical and pathological findings as in HE (6);
5. Administration of ammonium chloride to cultured astrocytes reproduces the pathological changes observed in HE (15,16);
6. Blood (14), CSF (17), and particularly brain (17) ammonia levels correlate well with the clinical state. At present, no other factor can better explain the clinical, pathological and neurochemical features of HE.

Ammonia exists either as the nonionized ammonia gas (NH_3), or as the protonated NH_4^+ ion. The nonionized form is a diffusible molecule (18) that readily crosses the blood-brain-barrier (19) whereas the ionized form is far less permeable. The pKa of ammonia is 9.0, so that at physiologic pH (7.4) over 98% of ammonia exists as the NH_4^+ ion. At alkaline pH, a greater amount of ammonia is present in the more diffusible form (NH_3) and is thus potentially more toxic.

Ammonia is largely generated from protein degradation and is principally detoxified in the liver by urea synthesis. In brain, however, ammonia is metabolized/detoxified into the electrophysiologically inert glutamine through the action of glutamine synthetase (GS), as the urea cycle is absent in brain (20). Increased brain and CSF levels of glutamine are among the neurochemical hallmarks of HE (19). The synthesis of glutamine is carried out in astrocytes (21) where GS is primarily localized (22). The glutamate required for this reaction is mostly derived by uptake from the extracellular space, whereas a smaller amount of ammonia may be utilized in the reductive amination of α -ketoglutarate, catalyzed by glutamate dehydrogenase (19). Glutamine is then released from astrocytes and taken up by nerve endings where it is converted to glutamate through the action of glutaminase (23). Glutamate released from nerve endings is later taken up by astrocytes thereby completing the so-called glutamate-glutamine cycle (24) (Fig. 1). The key components of the cycle, namely, glutamate uptake, glutamine synthesis, and glutamine release have all been convincingly shown in astrocyte cultures (25,26). As expected, treatment of cultured astrocytes with ammonia cause a reduction of glutamate and an increase in glutamine (21,27). As will be developed later, HE can be considered as a prototype disorder of the glutamate-glutamine cycle (*see* Fig. 1).

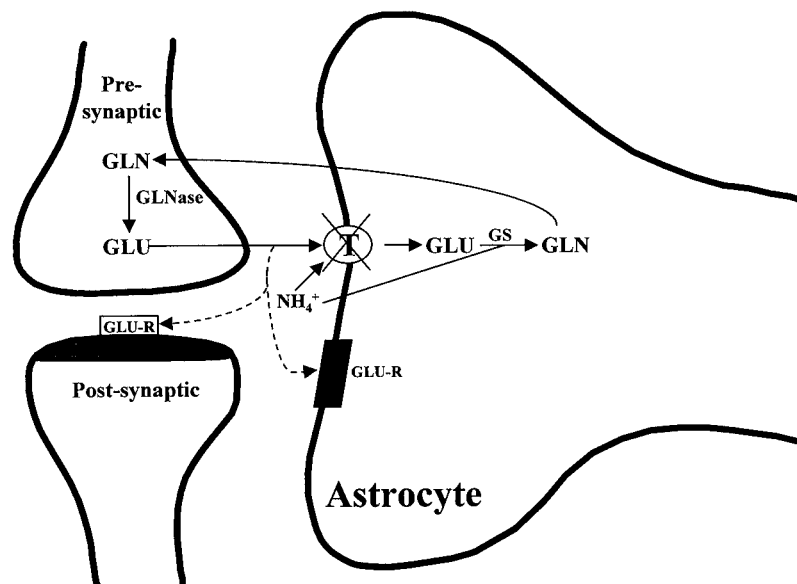


Fig. 1. Diagram depicting derangement in the glutamate-glutamine cycle and the role of ammonia-induced interference in glutamate uptake on glutamatergic neurotransmission. Failure of astrocytes to take up glutamate will lead to elevated levels in the synaptic space resulting in the activation of both neuronal and glial glutamate receptors (GLU-R). GLN, glutamine; GLNase, glutaminase; GLU, glutamate; GS, glutamine synthetase; T, glutamate transporter.

Ammonia is clearly a neurotoxin (19) which, in high concentrations (>1 mM), can result in seizures and death, while stupor and coma occur at lower concentrations. The fundamental mechanisms of ammonia toxicity are poorly understood. Views have ranged from altered bioenergetics, electrophysiological effects, altered intracellular pH, and effects on the membrane potential.

4.1.1. Energy Metabolism

The Bessmans (28) introduced the concept of energy failure as a factor in ammonia neurotoxicity. This view was based on the fact that the ammonia detoxification pathway (glutamine synthesis) consumes ATP. This view, however, has been controversial (see refs. 19,29 for reviews). In general, it appears that there are no acute losses of high energy metabolites; however, chronically, there is evidence of energy failure. One possible mechanism for such failure is the inability of the malate-aspartate shuttle to generate reducing equivalents due to an ammonia-induced fall in glutamate. This concept was originally introduced by Hindfelt et al. (30) and more recently supported by Ratnakumari and Murthy (31) and Faff-Michalak and Albrecht (32).

Another ammonia-induced energy demanding process is glutamate uptake which is coupled with Na⁺ influx. The latter is pumped out of the cell through the action of Na⁺, K⁺-ATPase. The activity of Na⁺, K⁺-ATPase is the major energy consuming process in the CNS (33), and the activity of this enzyme has been shown to be elevated in hyperammonemia (34–36). This view also goes along with the fact that energy demand in brain is tightly coupled to the recycling of glutamate to glutamine (37).

In view of the prominent role of astrocytes in HE, it is possible that energy failure may be compartmentalized in these cells. Yet, knowledge regarding bioenergetics in HE/ammonia toxicity in astrocytes is rudimentary. Fitzpatrick et al. (38) showed that 3 mM NH_4Cl decreased pyruvate oxidation in cultured astrocytes but had no effects on cultured neurons. Using relatively high concentrations of NH_4Cl (7.5–15 mM), Haghghat and McCandless (39) observed increase in lactate and decreases in ATP and phosphocreatine. Ammonia was also observed to impair the operation of the malate-aspartate shuttle in cultured astrocytes (40).

4.1.2. Electrophysiologic Effects

Effects on excitatory and inhibitory neurotransmission have been ascribed to ammonia, possibly due to inactivation of Cl^- extrusion pumps (41). Since NH_4^+ can substitute for K^+ (42), ammonia is also believed to interrupt nerve conduction (43). Ammonia has also been recently shown to depolarize astrocytes in culture (44), which can lead to inactivation of ionic conductance. This can contribute to cell swelling and disturbances in neurotransmitter uptake (see Subheading 4.4.).

4.1.3. pH

The importance of ammonia-induced changes in pH as a factor in ammonia neurotoxicity has received scant attention. This is surprising as ammonia has major effects on pH, and changes in pH have been shown to exert a profound effect on cell function by influencing the activity enzymes, the state of various ion channels, receptors and transporters (45,46), as well as by influencing lysosomal activity (47).

In most cells, ammonia ($\text{NH}_3 + \text{NH}_4^+$) produces a transient rise in pH (18). The situation in brain is not clear. Portacaval-shunted rats show increased pH in astrocytes (48). However, nuclear magnetic resonance (NMR) studies have either shown no change in pH (49), a rise (50), or a fall (51), presumably due to the generation of lactic acid. Although in high doses (20 mM) ammonia causes intracellular alkalosis in cultured astrocytes (52), in lower pathophysiologically relevant doses (0.1–5.0 mM), Nagaraja and Brookes (53) and ourselves (54) have found that ammonia causes intracellular acidification, suggesting the presence of NH_4^+ transporting channels in astrocytes. Similar NH_4^+ channels have been identified in kidney tubule cells (55) and *Xenopus* oocytes (56). The subsequent dissociation of NH_4^+ into NH_3 and H^+ would result in intracellular acidification.

4.1.4. Free Radicals

Oxidative stress is an evolving concept in the pathogenesis of HE/ammonia neurotoxicity (57). There is evidence of lipid peroxidation in cultured astrocytes after ammonia treatment (58). Ammonia was also recently shown to be capable of generating free radicals in cultured astrocytes (59). A dose-dependent response was observed, reaching its peak 2.5 min after exposure and declining to baseline levels by 15 min. The generation was blocked by the simultaneous addition of catalase and superoxide dismutase. Methionine sulfoximine (MSO) also blocked the production of free radicals, suggesting that glutamine may be involved in this process. Studies showing that antioxidants have a beneficial effect in experimental HE/hyperammonemia (60,61) and in patients with fulminant hepatic failure (62) further support the role of free radicals in HE.

The free radical nitric oxide (NO) has also been implicated in hyperammonemia/HE. Nitric oxide synthase (NOS) activity has been shown to be elevated in experimental

models of HE (63,64), and increased brain NO production was shown in portacaval shunted rats given ammonia infusions (65). Consistent with these observations is the ammonia-induced astrocyte uptake of arginine, the precursor for NO (66). The NOS inhibitor nitroarginine has been shown to attenuate ammonia toxicity (67)

4.1.5. General Effects of Ammonia on Astrocytes

In addition to effects on energy metabolism, membrane potential, pH and free radicals, ammonia has been shown to result in the morphological changes in astrocytes similar to those seen in HE; decreased GFAP content and mRNA levels; decreased glycogen content; altered protein phosphorylation; decreased cyclic AMP levels after stimulation with a β -adrenergic-receptor agonist; and decreased uptake of potassium, myo-inositol, and calcium. These and other effects on cultured astrocytes have been summarized (68). Similar, although not identical, findings have been reported by Reichenbach et al. (69) in cultured Müller (glial) cells of the neonatal rabbit retina. For review on the effect of ammonia on bulk-derived astrocytes from animals with acute HE, *see* ref. 70.

4.2. Glutamate Transport

The detoxification of ammonia consumes glutamate, so that abnormalities in glutamate metabolism and glutamatergic neurotransmission have long been suspected to contribute to the pathogenesis of HE (12,29). Indeed, total brain glutamate levels are decreased in various models of HE, in hyperammonemia, postmortem tissue from patients with HE, ammonia-treated brain slices, and ammonia-treated cultured astrocytes (reviewed in ref. 71). Interestingly, however, extracellular levels of glutamate are elevated in HE. Although increased release of glutamate has been reported in various models of HE and hyperammonemia, such "release" may be due to impaired glutamate uptake, either by nerve endings or by adjacent astroglial cells (*see* refs. 12,29 for further references).

Astrocytes are critically involved in glutamatergic neurotransmission (72,73) which is greatly dependent on glutamate uptake (74,75), and recent studies have shown a predominant role of glia in the clearing of glutamate after synaptic release (76). The uptake of glutamate is accomplished by various high-affinity, Na⁺-dependent transporters. Five subtypes of transporters have been cloned: EAAT1 (GLAST), EAAT2 (GLT-1), EAAT3 (EAAC1), EAAT4 and EAAT5. GLT-1 and GLAST are principally found in astrocytes whereas EAAC1 is present mainly in neurons. GLT-1 appears to be the main transporter *in vivo*. The status in astrocyte culture is less clear as some have found mainly GLAST with little to no GLT-1, whereas others have found significant amounts of GLT-1. EAAC1 is chiefly neuronal, most likely postsynaptic. EAAT4 and EAAT5 are additionally Cl⁻-dependent, and have been identified in neurons of the cerebellum and retina, respectively (for reviews, *see* refs. 77,78).

Treatment of cultured astrocytes with ammonia decreases glutamate uptake by 30–45% (71), which was reversible on discontinuation of ammonia treatment. Decreased uptake was also observed by Albrecht and colleagues (79) in bulk-isolated astrocytes derived from rats with acute liver failure. Depressed glutamate transport has likewise been described in synaptosomes derived from thioacetamide-treated rats (80), synaptosomes treated with ammonia (81), and hippocampal slices treated with sera and CSF from patients with HE (82).

The effect of ammonia on GLAST (EAAT1) mRNA has been examined in cultured astrocytes derived from cerebral cortex, striatum, and cerebellum (83). GLAST was

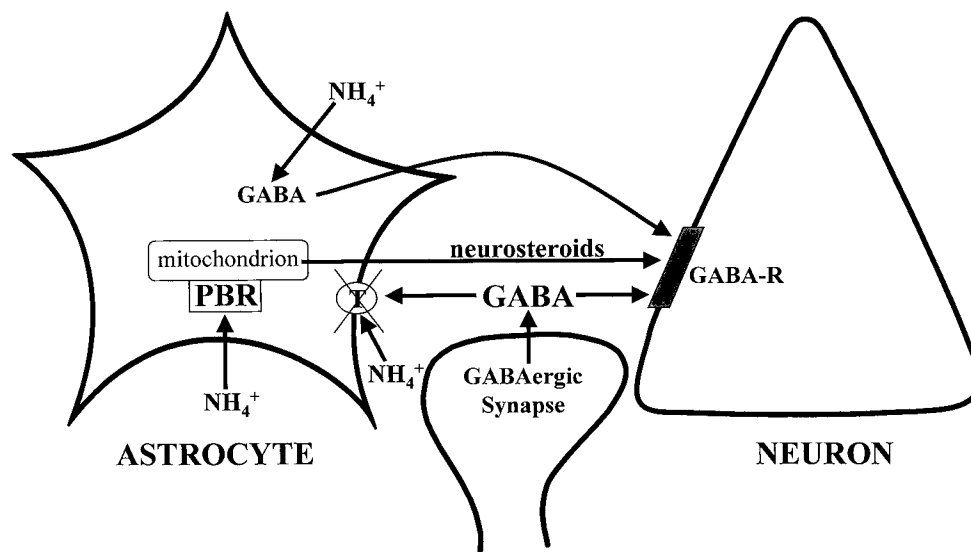


Fig. 2. Involvement of ammonia and astrocytes in enhanced GABAergic tone. Ammonia inhibits astroglial uptake of GABA and stimulates its release. This will result in elevated synaptic levels of GABA which can activate GABA_A receptors (GABA-R). Ammonia also upregulates the peripheral-type benzodiazepine receptor (PBR) leading to enhanced production of neurosteroids which positively modulate the GABA_A receptor.

selected for the study as it represents the principal transporter in cultured astrocytes (84,85). Decreased content of GLAST mRNA was observed in all regions, with the greatest effect noted in the striatum. The greater involvement of astrocytes derived from striatum is of interest because there is growing evidence of major involvement of the basal ganglia in HE (86), as evidenced by hyperintensities in the globus pallidus on MRI (4,5), which are likely because of the accumulation of manganese (11). In vivo studies have also shown a downregulation of the GLT-1 mRNA both in rats with hyperammonemia and with acute liver failure (29,87). As in the culture studies, the striatum showed the largest loss.

The mechanisms by which ammonia impairs glutamate uptake in astrocytes are not well understood. As noted, ammonia affects the transcription of transporter genes. Ammonia is known to cause cell swelling (*see Subheading 4.4*), membrane depolarization (44) nitric oxide production (65), and oxidative stress (57), which have all been shown to acutely impair astroglial glutamate uptake (88–91). Whether any or all of these factors are involved in the failure of astrocytes to take up glutamate is not known. Manganese, which has been implicated in HE (*see above*), was shown to decrease glutamate uptake, and had additive effects when combined with ammonia (92).

Failure of astrocytes to clear glutamate from the extracellular space may result in aberrant glutamatergic neurotransmission and excitotoxicity, as well as impairment in energy metabolism. In this regard, it is noteworthy that NMDA receptor antagonists are capable of ameliorating ammonia neurotoxicity (93,94). As glutamate receptors are located on astrocytes, excessive activation of these receptors may also lead to glial functional changes (95,96). Figure 2 depicts consequences of glutamate uptake failure.

4.3. GABAergic Neurotransmission

In the 1980s the increased GABAergic tone hypothesis dominated pathogenetic views of HE (97), and the fact that HE is associated with excessive neuroinhibition (stupor, coma) was certainly consistent with this concept. As the GABA hypothesis evolved, several factors were incriminated, including hypersensitivity of the GABA_A receptor, and/or excessive amounts of allosteric modulators of the GABA_A receptor (e.g., endogenous benzodiazepines; BZDs) (98–100). The mechanism for the increased BZD-like compounds in HE is not known. Strong support for the increased GABAergic tone hypothesis comes from studies showing that flumazenil, a GABA_A receptor antagonist, can reverse coma in some patients with chronic HE (101–103) and experimental animals (102).

Recent support for the GABA hypothesis has incorporated a role for ammonia (104,105). Ammonia has been shown to facilitate GABA-stimulated Cl⁻ currents (106), and to enhance the binding of benzodiazepines to the GABA_A receptor complex (107,108). Additionally, recent studies have shown that ammonia decreases the ability of astrocytes to take up GABA as well as enhance its release (109,110). The temporal response is in sharp contrast to that observed with the effect of ammonia on glutamate uptake; i.e., the effects on GABA uptake occurred within minutes, whereas the effects on glutamate uptake took at least one day to evolve. These effects of ammonia on astrocyte GABA transport may elevate synaptic levels of GABA sufficient to cause excessive GABAergic neurotransmission.

The peripheral-type benzodiazepine receptor (PBR) further links the GABA hypothesis to astrocytes. In contrast to the central benzodiazepine receptor, the PBR is primarily located on the outer mitochondrial membrane (111). The PBR also has a different rank order of ligand binding affinities than the “central” receptor. In the CNS, PBRs are not found on neurons but rather on astrocytes (112,113) and microglia (114,115). Although the function of the PBR is not completely understood, its best known function is steroidogenesis as it serves to transfer cholesterol from the outer to the inner mitochondrial membrane (116). By the action of cytochrome P450_{scc} cholesterol is subsequently converted to pregnenolone, the parent compound for all neurosteroids. The steroid products are predominantly derivatives of progesterone, some of which (e.g., tetrahydroprogesterone, THP; tetrahydrodeoxycorticosterone, THDOC) are among the most potent positive modulators of the GABA_A receptor known (for review, see ref. 117).

Ammonia has been shown to upregulate the PBR in cultured astrocytes (118). Butterworth and colleagues have shown increased numbers of PBR receptors in human postmortem tissue from encephalopathic patients (119), and increased binding sites and mRNA levels were found in portacaval-shunted rats (120,121). Increased numbers of PBR binding sites were similarly identified in hyperammonemic and thioacetamide-treated mice (122). Interestingly, manganese, another purported HE-related toxin has been shown to upregulate the PBR (123). We have also shown that animals with HE/hyperammonemia had elevated levels of THDOC, THP, and pregnenolone (124).

Neurosteroids and benzodiazepines may also exert direct effects on astrocytes that are potentially relevant to HE. Some neurosteroids decrease the ability of astrocytes to take up glutamate, GABA and K⁺. They also exert behavioral and neuropathologic

changes similar to HE (Alzheimer type II change). These studies have been summarized in a recent publication (124).

The involvement of the PBR and neurosteroids in the pathogenesis of HE may have therapeutic implications. Treatment with PK 11195, a putative antagonist of the PBR, significantly attenuated ammonia toxicity (125). Pregnenolone sulfate whose electrophysiological effects are opposite to that of allopregnanolone (126) also had a protective effect, although of lesser magnitude (125).

4.4. Astrocyte Swelling

Brain edema/swelling leading to increased intracranial pressure is a major cause of death (20–70%) in patients with FHF (127). There is currently no satisfactory treatment for this condition other than liver transplantation. Although the basis for the intracranial hypertension is not clear, astroglial swelling plays an important role in this process (7,128,129).

There are several consequences of astrocyte swelling. Most important is the mass effect resulting in increased intracranial pressure and brain herniation. Swelling also results in cell depolarization (130), thereby interfering with the cell's ability to maintain ionic gradients and uptake of neurotransmitters. The reduction in the size of the extracellular space following astrocyte swelling may elevate extracellular ionic concentrations which can affect neuronal excitability (131). Astrocyte swelling may also compress capillaries, contributing to a reduction in cerebral blood flow (132). As suggested by Häussinger (133), even small volume changes in astrocytes may affect cell function similar to those previously shown in cultured hepatocytes (134).

The mechanism of astroglial swelling in FHF is not completely understood. Clearly ammonia plays a major role as it has been shown to cause swelling of astrocytes in culture (135), brain slices (136,137), and in vivo (138–140). Furthermore, the extent of intracranial pressure correlates well with arterial ammonia levels in patients with acute liver failure (141). Brusilow and Traystman (142) have proposed that the generation of glutamine from glutamate and ammonia by the action of glutamine synthetase (GS) may create a sufficient osmotic load to create astroglial swelling. Support for this view was derived from studies showing that treatment of hyperammonemic rats with MSO, an inhibitor of GS, reduced the amount of brain edema (143,144) and diminished the extent of astrocyte swelling (140). Similarly, ammonia-induced swelling of cultured astrocytes could be abolished by treatment with MSO (145). Additionally, MSO induces a massive release of glutamine from glial cells (146) which may contribute to its beneficial effects in HE. Thus, although the synthesis of glutamine is generally regarded as the principal means of detoxifying ammonia, this "protective" process also causes glial swelling and brain edema. It appears that the detoxification of ammonia occurs at the expense of developing astroglial swelling.

It is possible that other osmolytes may contribute to glial swelling. Ammonia-treated astrocytes showed an elevation in glutathione (GSH) levels (147,148). The magnitude of GSH increase observed in this study was comparable to elevations in glutamine occurring after ammonia treatment (27). As in the case of glutamine, GSH concentration in astrocytes is also in millimolar range (149), and there is evidence that it may also act as an osmolyte (150,151). It is thus possible that part of the swelling after ammonia treatment may be due to GSH. This premise is supported by a recent report

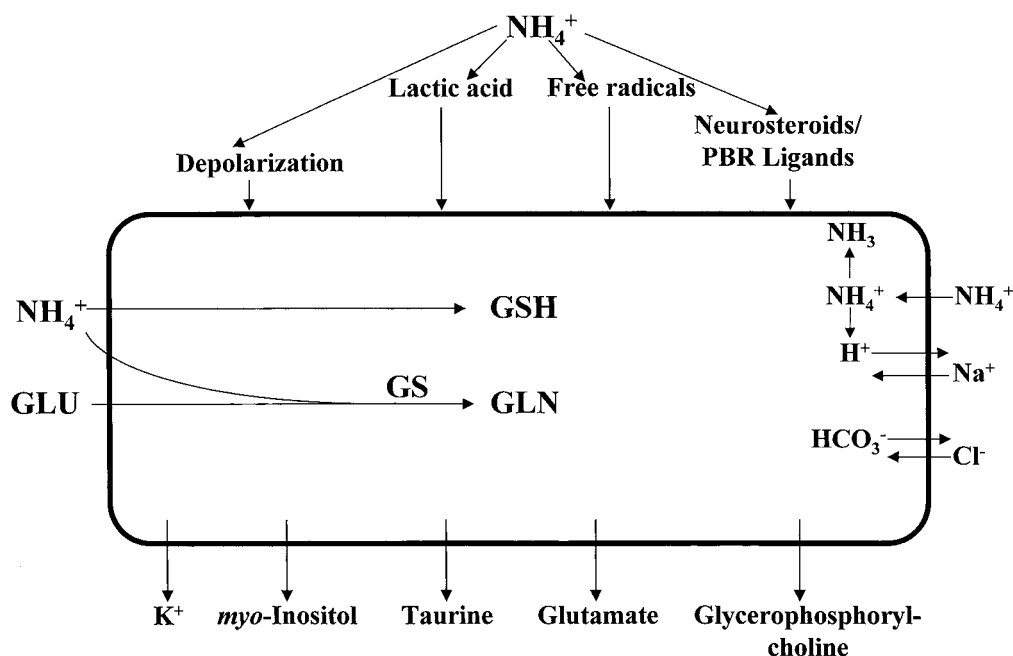


Fig. 3. Schematic illustration of mechanisms and consequences of ammonia-induced astroglial swelling. Factors known to be associated with ammonia, and known to cause swelling, are shown on top. Additionally, ammonia-mediated increase in glutamine and glutathione (GSH), which can act as osmolytes, are shown. Also, the influx of NH_4^+ through NH_4^+/K^+ channels will result in the influx of NaCl along with obliged water. As part of the volume regulatory events, inorganic (K^+) and organic (*myo*-inositol, taurine, glutamate, and glycerophosphorylcholine) osmolytes are released. These released osmolytes may have potent effects on CNS function. GLN, glutamine; GLU, glutamate; GS, glutamine synthetase; PBR, peripheral-type benzodiazepine receptor.

noting that BSO, which inhibits γ -glutamylcysteine synthetase activity (152), the GSH catabolizing enzyme, decreased ammonia-induced astrocyte swelling (147).

As noted previously, there is emerging data on the potential role of free radicals in ammonia toxicity. Since free radicals may contribute to astrocyte swelling (153,154), it is possible that ammonia-induced oxidative stress may also be involved in astrocyte swelling. Similarly, ammonia has been shown to cause membrane depolarization in cultured astrocytes (44). Such depolarization can lead to passive, Donnan-mediated KCl and water fluxes into cells (155). Also, the unidirectional entry of NH_4^+ into astrocytes leads to an intracellular acidosis (53); this would stimulate the Na^+/H^+ exchanger which, along with activation of the $\text{HCO}_3^-/\text{Cl}^-$ exchanger, will result in NaCl and obliged water entering the cell (156,157). The accumulation of lactic acid in HE/hyperammonemia (158–160) may also be a factor as lactic acid has been shown to cause glial swelling (161,162). Additionally, PBR ligands and neurosteroids may contribute to astrocyte swelling (163). Potential mechanisms responsible for ammonia-mediated astroglial swelling are summarized in figure 3.

In response to swelling, a number of volume regulatory systems become activated (164), particularly the release of inorganic (K^+) and organic (glutamate, taurine, *myo*-

inositol, glycerophosphorylcholine) osmolytes (165). The release of these factors by swollen astrocytes, however, may have profound effects on CNS excitability and function.

myo-Inositol is an important regulatory organic osmolyte (166) and is one of the main organic osmolytes in glial cells (167). Brain *myo*-inositol levels has been shown to be decreased in chronic HE (133,168–170). Treatment of astrocytes with ammonium chloride (5 mM) decreased *myo*-inositol uptake, increased its release and decreased its intracellular content (171,172). As *myo*-inositol is important in the metabolism of various second messenger and lipids, loss of this metabolite may have major consequences on cell function.

Brain taurine levels are decreased in rats six weeks after a portacaval anastomosis (170). This decrease is likely due to increased release as ammonia has been shown to stimulate taurine release in cultured astrocytes (173), and *in vivo* (174). While this release would have volume regulatory effects, taurine has inhibitory neuromodulatory effects (175) which could exacerbate the neuroinhibition in HE. On the other hand, taurine may exert a neuroprotective effect through its known antioxidant properties (176). This may be particularly pertinent as there is recent evidence of excessive free radical formation in hyperammonemia (*see Subheading 4.*).

5. CONCLUDING REMARKS

The pathogenesis of HE and the mechanism of ammonia neurotoxicity remain enigmatic. Nevertheless, it is clear that astrocytes are a target of ammonia toxicity. Such toxicity results in a “primary gliopathy” leading to a disturbance in glial-neuronal interactions/trafficking, disruption of the glutamate-glutamine cycle, bioenergetic failure and potential abnormalities in glutamatergic and GABAergic neurotransmission, as well as in cell swelling. The initial event triggering these phenomena is not clear. A number of likely candidates are emerging, including oxidative stress and changes in membrane potential and pH.

This review has presented evidence that abnormalities in glutamatergic, and GABAergic neurotransmission are key considerations in HE. More importantly, it has attempted to integrate available data implicating ammonia neurotoxicity as a major factor in these neurotransmitter defects. Inadequate astroglial glutamate transport may lead to abnormalities in glutamatergic neurotransmission, and possible excitotoxic injury. Although the role of GABA in HE has been controversial, ammonia-induced decrease in astrocytic GABA uptake and increased release, as well as enhanced production of neurosteroids known to have a positive modulatory effect on the GABA_A receptor are supportive of this hypothesis.

Astrocyte swelling in fulminant hepatic failure is a major clinical problem. The mechanisms for such swelling are unclear but likely include the generation of excessive osmolytes, chiefly glutamine and possibly glutathione. Additionally, oxidative stress and abnormalities in intracellular pH and membrane potential can also disrupt in ionic homeostasis and lead to cell swelling.

A fuller understanding of the role of astrocytes in HE and ammonia toxicity will undoubtedly yield critical information on glial function and glial-neuronal interactions, and hopefully generate novel therapeutic strategies.

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Neuroglia in the Aging Brain

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With the proportion of the aged in the population rapidly increasing, especially those 80 and beyond, it has become imperative to understand how to prevent and treat the disabilities and diseases of old age and to prolong and improve functional ability. The biology of neuroglia has thus emerged as one of the most exciting research areas in the neurosciences. In *Neuroglia in the Aging Brain*, Jean de Vellis and a distinguished panel of internationally recognized neuroscientists comprehensively review the involvement of and changes in glial cells both during the normal aging process and in the major disorders of old age. Topics range from the cellular and molecular changes that occur with aging—especially aging-associated gliosis in astrocytes and its relation to neuronal injury and repair—to neuron–glia intercommunication. The contributors show how glial signals may be modulated by hormones, growth factors, neurotransmitters, intracellular metabolism, and intercellular exchanges, as well as by aging of the blood–brain barrier. Also discussed is the role of the neuroglia, especially of the astrocytes and microglia, in the etiology of several neurodegenerative diseases, as well as the possibilities for therapeutic interventions in glial responses, and indirectly on neurons.

Illuminating and multidisciplinary, *Neuroglia in the Aging Brain* provides today’s neuroscientists and neuropathologists with powerful new insights into how glial cells are involved in the pathogenesis of major aging disorders and how they may be used therapeutically to encourage neuronal self-renewal.

Features

- Discusses the latest thinking on using glial cells to promote neuronal self-renewal
- Examines the role of neuroglia in causing neurodegenerative diseases
- Proposes therapeutic interventions based on glial responses
- Reviews the latest findings on the cellular and molecular changes that occur with aging

Contents

Part I. Cellular and Molecular Changes of Aged and Reactive Astrocytes. Neuromorphological Changes in Neuronal and Neuroglial Populations of the Cerebral Cortex in the Aging Rat: *Neurochemical Correlations*. Diversity in Reactive Astrocytes. Astrocytic Reaction After Traumatic Brain Injury. **Part II. Neuron–Glial Intercommunication.** Astrocytes *In Situ* Exhibit Functional Neurotransmitter Receptors. Glia and Extracellular Space Diffusion Parameters in the Injured and Aging Brain. Intercellular Diffusional Coupling between Glial Cells in Slices from the Striatum. Glial Cell Involvement in Brain Repair and the Effects of Aging. ATP Signaling in Schwann Cells. **Part III. Neurotrophins, Growth Factors, and Neurohormones in Aging and Regeneration.** Gliosis Growth Factors in the Adult and Aging Rat Brain. Role of Fibroblast Growth Factor-2 in Astroglial Gliosis. Trophins as Mediators of Astrocyte Effects in the Aging and Regenerating Brain. Responses in the Basal Forebrain Cholinergic System to Aging. Effects of Estrogens and Thyroid Hormone on Development

and Aging of Astrocytes and Oligodendrocytes. **Part IV. Metabolic Changes.** Neurotoxic Injury and Astrocytes. Ammonium Ion Transport in Astrocytes: *Functional Implications*. **Part V. Astrocytes and the Blood–Brain Barrier in Aging.** Molecular Anatomy of the Blood–Brain Barrier in Development and Aging. The Blood–Brain Barrier in the Aging Brain. Astrocytes and Barrier-provided Microvasculature in the Developing Brain. **Part VI. Astrocytes in Neurodegenerative Diseases.** Microglial and Astrocytic Reactions in Alzheimer’s Disease. Activated Neuroglia in Alzheimer’s Disease. Reactive Astroglia in the Ataxic Form of Creutzfeldt–Jakob Disease: *Cytology and Organization in the Cerebellar Cortex*. Ischemic Injury, Astrocytes, and the Aging Brain. Glial–Neuronal Interactions during Oxidative Stress: *Implications for Parkinson’s Disease*. Astrocytic Changes Associated with Epileptic Seizures. Synaptic and Neuroglial Pathobiology in Acute and Chronic Neurological Disorders. Astrocytes and Ammonia in Hepatic Encephalopathy. Index.

