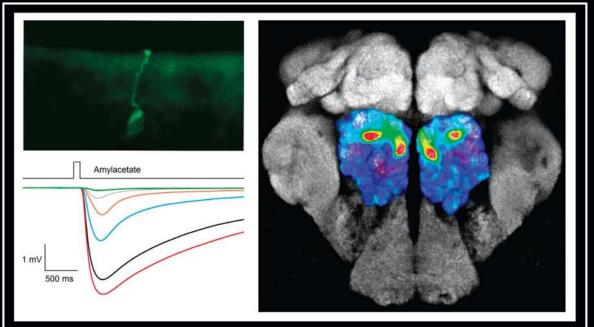


# The Neurobiology of Olfaction



# Edited by Anna Menini



**FRONTIERS IN NEUROSCIENCE** 

# The NEUROBIOLOGY of OLFACTION

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#### The Neurobiology of Olfaction

Anna Menini, Ph.D., Neurobiology Sector International School for Advanced Studies, (S.I.S.S.A.), Trieste, Italy

# The NEUROBIOLOGY of OLFACTION

Edited by Anna Menini



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CRC Press Taylor & Francis Group 6000 Broken Sound Parkway NW, Suite 300 Boca Raton, FL 33487-2742

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Printed in the United States of America on acid-free paper 10 9 8 7 6 5 4 3 2 1

International Standard Book Number: 978-1-4200-7197-9 (Hardback)

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The neurobiology of olfaction / editor, Anna Menini.
p.; cm. -- (Frontiers in neuroscience)
Includes bibliographical references and index.
ISBN 978-1-4200-7197-9 (hardcover : alk. paper)
1. Smell. 2. Neurobiology. I. Menini, Anna. II. Series: Frontiers in neuroscience (Boca Raton, Fla.)
[DNLM: 1. Olfactory Perception--physiology. 2. Odors. 3. Olfactory Bulb--physiology. WV 301
N494 2009]

QP458.N48 2009 612.8'6--dc22

2009017977

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# Series Preface

Our goal in creating the Frontiers in Neuroscience Series is to present the insights of experts on emerging fields and theoretical concepts that are, or will be, in the vanguard of neuroscience. Books in the series cover genetics, ion channels, apoptosis, electrodes, neural ensemble recordings in behaving animals, and even robotics. The series also covers new and exciting multidisciplinary areas of brain research, such as computational neuroscience and neuroengineering, and describes breakthroughs in classical fields like behavioral neuroscience. We hope every neuroscientist will use these books in order to get acquainted with new ideas and frontiers in brain research. These books can be given to graduate students and postdoctoral fellows when they are looking for guidance to start a new line of research.

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Sidney A. Simon, Ph.D. Miguel A.L. Nicolelis, M.D.,Ph.D. Series Editors

# Preface

Our knowledge of the neurobiology of olfaction has increased over the past few years, urging the publication of an up-to-date book on the subject. In this book, authors from some of the best laboratories around the world present a survey of the current status of knowledge and research about olfaction. The book is highly interdisciplinary, covering contents from genetics to behavior and from nematodes to humans. It includes chapters about odor coding from odorant receptors to cortical centers, development, and neurogenesis, highlighting common principles in different species. Each chapter provides an overview of a topic with an emphasis on the most recent discoveries and an exhaustive list of references of reviews and original articles for students or scientists interested in further readings.

The new knowledge about olfaction has been obtained employing several techniques, for which the reader is referred to the relevant chapters in the previously published book, *Methods in Chemosensory Research* (Eds. Simon and Nicolelis).

I wish to especially thank Sid Simon for inviting me to embark on the interesting but also troublesome adventure of preparing this volume. I am particularly grateful to Manuela Schipizza-Lough, who enthusiastically contributed to the precise completion of this work, and I am indebted to Andrea Tomicich for professionally solving several technical problems.

Finally, a special thanks to John W. Moore for his continuous encouragement and support along my scientific career.

Anna Menini Trieste, Italy

# Editor

**Anna Menini**, PhD, is professor of physiology in the Sector of Neurobiology at the International School of Advanced Studies, Scuola Internazionale Superiore di Studi Avanzati (SISSA), Trieste, Italy, where she also serves as coordinator of the PhD program in neuroscience. Born in Rapallo, Italy, she received a PhD in physics from the University of Genova, Italy, and after postdoctoral work at Duke University Medical Center, Durham, NC, she joined the Institute of Cybernetics and Biophysics of the National Research Council in Genova.

In 2002, Dr. Menini was appointed full professor at SISSA, where she also belongs to the SISSA. Research Unit of the Italian Institute of Technology. She is interested in understanding how the brain represents the external world, with a focus on the molecular mechanisms that transform the chemical odorant signal into the electrical messages that are transmitted to the brain.

# Contributors

# **Brice Bathellier**

Research Institute of Molecular Pathology Vienna, Austria

# Ottorino Belluzzi

Dipartimento di Biologia ed Evoluzione Università degli Studi di Ferrara

# and

Istituto Nazionale di Neuroscienze Sezione di Ferrara Ferrara, Italy

# Peter A. Brennan

Department of Physiology and Pharmacology University of Bristol Bristol, United Kingdom

# Anne L. Calof

Department of Anatomy and Neurobiology Department of Developmental and Cell Biology Center for Complex Biological Systems University of California Irvine, California

# **Alan Carleton**

Laboratory of Sensory Perception and Plasticity Department of Neurosciences, Faculty of Medicine University of Geneva Geneva, Switzerland

# Michael Y. Chao

Department of Biology California State University San Bernardino San Bernardino, California

# C. Giovanni Galizia

Neuroscience Universität Konstanz Konstanz, Germany

# Kimberly K. Gokoffski

Department of Anatomy and Neurobiology Department of Developmental and Cell Biology Center for Complex Biological Systems University of California Irvine, California

# Daniela C. Gonzalez-Kristeller

Departamento de Bioquímica, Instituto de Química Universidade de São Paulo São Paulo, Brazil

# **Charles A. Greer**

Departments of Neurosurgery and Neurobiology Yale University School of Medicine New Haven, Connecticut

# **Olivier Gschwend**

Laboratory of Sensory Perception and Plasticity Department of Neurosciences, Faculty of Medicine University of Geneva Geneva, Switzerland

# Luciana M. Gutiyama

Departamento de Bioquímica, Instituto de Química Universidade de São Paulo São Paulo, Brazil

# Anne C. Hart

Center for Cancer Research Massachusetts General Hospital Charlestown, Massachusetts

# S. Shuichi Haupt

Research Center for Advanced Science and Technology The University of Tokyo Tokyo, Japan

# Piper L.W. Hollenbeck

Department of Anatomy and Neurobiology Department of Developmental and Cell Biology Center for Complex Biological Systems University of California Irvine, California

# Ryohei Kanzaki

Research Center for Advanced Science and Technology The University of Tokyo Tokyo, Japan

# Shimako Kawauchi

Department of Anatomy and Neurobiology University of California Irvine, California

# Tomoki Kazawa

Research Center for Advanced Science and Technology The University of Tokyo Tokyo, Japan

# Takashi Kurahashi

Department of Frontier Biosciences Osaka University Osaka, Japan

# Arthur D. Lander

Department of Developmental and Cell Biology Center for Complex Biological Systems University of California Irvine, California

# **Minghong Ma**

Department of Neuroscience University of Pennsylvania School of Medicine Philadelphia, Pennsylvania

# **Bettina Malnic**

Departamento de Bioquímica, Instituto de Química Universidade de São Paulo São Paulo, Brazil

# Ivan Manzini

Department of Neurophysiology and Cellular Biophysics University of Göttingen Göttingen, Germany

## Anna Menini

Neurobiology Sector International School for Advanced Studies S.I.S.S.A. Unit of the Italian Institute of Technology Trieste, Italy

## Alexandra M. Miller

Department of Neurosurgery Yale University School of Medicine New Haven, Connecticut

# **Anne-Marie Mouly**

Laboratoire Neurosciences Sensorielles, Comportement, Cognition CNRS-Université de Lyon Lyon, France

# Shigehiro Namiki

Research Center for Advanced Science and Technology The University of Tokyo Tokyo, Japan

# **Simone Pifferi**

Neurobiology Sector International School for Advanced Studies S.I.S.S.A. Unit of the Italian Institute of Technology Trieste, Italy

# Angela Pignatelli

Dipartimento di Biologia ed Evoluzione Università degli Studi di Ferrara

#### and

Istituto Nazionale di Neuroscienze Sezione di Ferrara Ferrara, Italy

# Arundhati Ray

Department of Neurosurgery Yale University School of Medicine New Haven, Connecticut

# Robert L. Rennaker

Neural Engineering Laboratory School of Aerospace and Mechanical Engineering University of Oklahoma Norman, Oklahoma

#### Contributors

Silke Sachse Max Planck Institute for Chemical Ecology Jena, Germany

**Takeshi Sakurai** Research Center for Advanced Science and Technology The University of Tokyo Tokyo, Japan

## **Rosaysela Santos**

Department of Anatomy and Neurobiology Department of Developmental and Cell Biology University of California Irvine, California

# **Detlev Schild**

Department of Neurophysiology and Cellular Biophysics University of Göttingen Göttingen, Germany

# Gordon M. Shepherd

Department of Neurobiology Yale University School of Medicine New Haven, Connecticut

#### **Regina Sullivan**

Emotional Brain Institute Nathan Kline Institute for Psychiatric Research Child and Adolescent Psychiatry New York University Langone Medical Center New York, New York Helen B. Treloar Department of Neurosurgery Yale University School of Medicine New Haven, Connecticut

# Matt Wachowiak

Departments of Biology and Biomedical Engineering Center for Neuroscience Boston University Boston, Massachusetts

#### **Donald A. Wilson**

Emotional Brain Institute Nathan Kline Institute for Psychiatric Research Child and Adolescent Psychiatry New York University Langone Medical Center New York, New York

# Hsiao-Huei Wu

Department of Anatomy and Neurobiology University of California Irvine, California

#### and

Center for Stem Cell Biology Vanderbilt University School of Medicine Nashville, Tennessee

# 1 From Odors to Behaviors in *Caenorhabditis elegans*

Anne C. Hart and Michael Y. Chao

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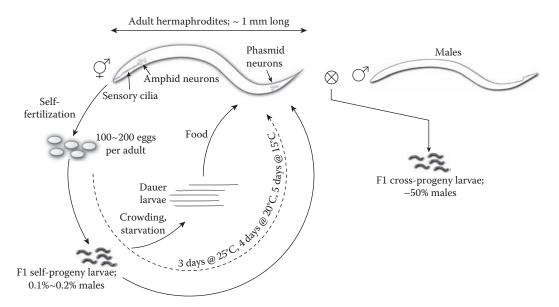
# **1.1 INTRODUCTION**

Over 30 years ago, Nobel laureate Sydney Brenner recognized that an intellectually straightforward strategy to delineate the basic principles in neurobiology is to utilize a model

organism with a nervous system that is simple enough to lend itself to anatomical, cellular, genetic, and molecular analysis, yet be complex enough that lessons learned in that organism would give us insight into general principles of neural function. The humble organism he chose, the nematode *Caenorhabditis elegans*, is now one of the most thoroughly characterized metazoans, particularly in terms of its nervous system. One of Brenner's motivations in adapting *C. elegans* as a model organism was to understand the totality of the molecular and cellular basis for the control of animal behavior (Brener 1988). In this chapter, we review what is arguably the best-studied aspect of *C. elegans* behavior: response to chemical stimuli. The *C. elegans* neurobiology literature can be intimidating for the uninitiated; we attempt to limit the use of "worm jargon" in this review. For a more *C. elegans*-centric review, we refer you to other excellent sources (Bargmann 2006).

#### **1.1.1 CAENORHABDITIS ELEGANS BASICS**

*C. elegans* are hermaphroditic, free-living nematodes. The adult hermaphrodite is ~1 mm in length. Each hermaphrodite produces oocytes and sperm, can internally self-fertilize, and has several hundred self-progeny that reach reproductive maturity in 3-5 days, depending on cultivation temperature. Hermaphrodites cannot cross-fertilize, but they can mate with males, resulting in numerous cross-progeny. In normal populations, *C. elegans* males appear at a 0.1-0.2% frequency (Figure 1.1). The ability to set up genetic crosses, relatively large brood sizes, and rapid generation time greatly facilitates forward genetic analysis in *C. elegans*. Much of what is known about olfaction in *C. elegans* is based on classical genetic studies of mutant strains defective in chemosensory response.



**FIGURE 1.1** Schematic of the *C. elegans* life cycle. Adult hermaphrodite *C. elegans* typically produce 100–200 self-fertilized eggs. Under normal conditions, larvae go through four larval stages, called L1–L4 (not shown); the time it takes to reach adulthood depends on cultivation temperature. If larvae develop under crowded or starved conditions, they enter an alternate larval stage, called dauer. If conditions permit, dauer larvae can exit the dauer stage and continue to develop into reproductive adults. In a normal self-cross, males are rare (0.1-0.2% of total F1 progeny). If a male is crossed to a hermaphrodite, the hermaphrodite effectively functions as a female, and males appear at the expected Mendelian ratio. This sexual dimorphism allows for genetic analysis in *C. elegans* via classical genetic mapping and double mutant analysis.

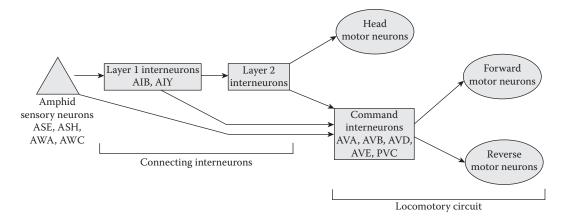
In the laboratory, *C. elegans* is reared in agar dishes seeded with *Escherichia coli* bacteria, which serve as a food source for the animals. On flat agar surfaces, *C. elegans* lies on its side and, when unperturbed, spends most of its time crawling forward with dorsal–ventral sinusoidal body bends at a rate of about 0.5 mm/s, punctuated by occasional spontaneous reversals in locomotion direction. *C. elegans* can initiate turns via omega bends, where the anterior end of the animal bends toward the posterior end and forms the shape of the Greek letter  $\Omega$ , or by altering the amplitude of body bends (Gray et al. 2005). This mode of locomotion on flat agar surfaces is probably very artificial. Under conditions designed to simulate soil (presumably similar to the natural environment of *C. elegans*), the locomotory pattern of *C. elegans* changes to a more efficient hybrid mode between crawling and swimming, which is up to 10 fold faster than locomotion on surfaces (Park et al. 2008; Lockery et al. 2008).

#### 1.1.2 CHEMOSENSORY NEUROANATOMY

#### 1.1.2.1 The Sensory Nervous System of Caenorhabditis elegans

C. elegans follows an almost invariant developmental pattern. Each adult hermaphrodite has precisely 959 somatic cells, of which almost one-third (302) are neurons. Each neuron is designated with a unique name, typically consisting of three or four letters (e.g., AWA, AWC). The synaptic connectivity of all 302 neurons has been mapped out using serial electron microscopy reconstruction and is remarkably reproducible between animals (Chen et al. 2006; White et al. 1986), thus making C. elegans the only metazoan with a completely characterized neuroanatomy down to the synaptic level. Among neurons, 16 pairs of anatomically bilaterally symmetric neurons (i.e., 32 neurons or ~10% of the nervous system) have been confirmed or inferred to be chemosensory based on functional studies or anatomy, as described below. These chemosensory neurons respond to a wide variety of soluble and volatile odorants. In a survey of volatile organic compounds, C. elegans exhibited either attraction or repulsion to 50 out of 120 compounds tested (Bargmann et al. 1993). C. elegans chemosensory neurons synapse directly or indirectly onto a set of command interneurons (named AVA, AVB, AVD, AVE, and PVC) that control forward or backward locomotion through synapses with motor neurons that control body wall muscles (Chalfie et al. 1985; Vonstetina et al. 2005) (Figure 1.2). Additional interneurons relevant to chemosensory behavior are discussed in Section 1.4.

C. elegans has four types of visible chemosensory organs: the amphid, phasmid, inner labial, and outer labial organs (Figure 1.3). Each consists of two support cells, called sheath and socket cells, which form a pore through which sensory neuron endings are exposed to the external milieu (White et al. 1986; Ward et al. 1975). The pores are bilaterally or quadrilaterally symmetric and contain a poorly characterized substance probably reminiscent of mucosal secretions in vertebrates. The two amphid pores are located at the tip of the head and play a critical role in response to attractive chemical stimuli. Each contains the sensory endings of 11 chemosensory neurons and one thermosensory neuron (AFD). The ciliated sensory endings of these bilaterally symmetric chemosensory neurons are located in the amphid pore (ADL, ADF, ASE, ASG, ASH, ASI, ASJ, and ASK) or embedded in the sheath cell (AWA, AWB, and AWC neurons; also called wing neurons). The phasmid pores are structurally similar to the amphids, but are smaller, located behind the anus near the tail, and contain the sensory endings of the PHA and PHB neurons (Hall and Russell 1991; White et al. 1986; Ward et al. 1975). These neurons have been implicated in chemosensory avoidance. The fourfold symmetric inner and outer labial pores are located in the labia surrounding the mouth of C. elegans. Based on anatomical evidence, neurons associated with these organs (IL1, IL2, OLL, OLQ) probably play a role in sensory response. Laser ablation of the inner labial IL2 neurons has not resulted in any apparent defects in response to chemosensory cues thus far (Bargmann et al. 1993), and the outer labial OLQ neurons are required for mechanosensory response to nose touch (Kaplan and Horvitz 1993). The role of the other labial neurons remains unclear.



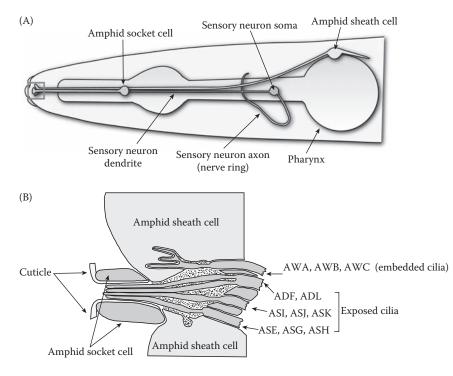
**FIGURE 1.2** Simplified wiring diagram of the *C. elegans* chemosensory nervous system. Most amphid sensory neurons synapse to the locomotory circuit via multiple layers of connecting interneurons. However, some sensory neurons (e.g., ASH) synapse directly onto the command interneurons, and, similarly, some layer 1 interneurons also synapse directly onto the command interneurons. There are also substantial synapses between neurons in the same layers of organization, as well as gap junction connections (not shown). In this diagram, only neurons that have been discussed in some detail in this chapter are labeled. For a complete list of chemosensory neurons, see Table 1.1. (Adapted from Gray, J.M. et al. *Proc. Natl. Acad. Sci. USA*, 102, 3184–91, 2005.)

Sensory neuron ciliated endings are the probable sites of olfactory reception in *C. elegans*. Candidate seven transmembrane domain chemosensory receptors and other proteins implicated in the initial steps of olfactory response, localize to the sensory endings. For example, ODR-10, which is required specifically for response to diacetyl, localizes to the tip of the AWA ciliated endings (Sengupta et al. 1996). Early cellular ablation studies with a laser microbeam suggest that specific amphid sensory neurons are required for response to either attractive or repulsive stimuli, but not both (Bargmann and Avery 1995; Bargmann et al. 1993). More recent genetic studies suggest that altering neural activity in sensory neurons can alter odor preference (Tsunozaki et al. 2008; also see below).

# 1.1.2.2 Functions of Specific Chemosensory Neurons

The role of specific *C. elegans* neurons in behavior has classically been defined using cellular ablation with a laser microbeam (Bargmann and Avery 1995). Briefly, an early larval stage animal is anesthetized and neurons are identified under high power differential interference contrast (DIC) microscopy. A nitrogen pulsed dye laser is focused through the objective of the microscope to heat and kill targeted cell nuclei. Animals are transferred to a Petri dish to recover and develop into adulthood, upon which they can be tested for behavioral responses to odorants. This powerful approach has allowed the matching of specific odorants to specific chemosensory neurons.

Assignment of neurons to specific behavioral responses by laser ablation is augmented by two other strategies: cellular rescue studies and optical imaging. In the first strategy, a gene normally expressed in sensory neurons that is required for response to a stimulus is identified. Heterologous promoters are used to drive cDNA expression in specific subsets of neurons in mutant animals. If cDNA expression rescues the behavioral defect in mutant animals, then the gene is required in those sensory neurons and those neurons are important for behavioral response. The second strategy relies on the optical detection of stimulus-induced activity in neurons of the live animals (see Section 1.1.3.3). Immediate evoked responses in sensory neurons suggest they play a role in behavioral response. Although none of these approaches are definitive in isolation, in combination they have allowed *C. elegans* researchers to define distinct roles for specific neurons.



**FIGURE 1.3** The structure of the amphid sensilla. (A) The soma of amphid sensory neurons are arranged around the pharynx, which is the feeding organ of *C. elegans*. The axons synapse with interneurons in a structure called the nerve ring. The dendrites extend anteriorly. Only one sensory neuron is shown for clarity. The region indicated by the red box is shown enlarged in panel B. (B) The sensory endings of the so-called wing neurons (AWA, AWB, and AWC) are embedded within the amphid sheath cell. The cilia of the thermosensory AFD neurons (not shown) are also embedded in the sheath cell. Other amphid sensory neurons have exposed cilia. ([A] Redrawn from Hall, D.H. and Z.F. Altun, *C. elegans Atlas*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2008. [B] Adapted from Perkins, L.A. et al *Dev. Biol.*, 117, 456–87, 1986.)

In general, the ASE neurons detect soluble attractants, whereas the AWA and AWC neurons detect volatile attractants (Bargmann et al. 1993). The ASH, ADL, and AWB neurons detect volatile repellants (Chao et al. 2004; Troemel et al. 1995). The ASH neurons also detect soluble repellants, including heavy metals such as Cu<sup>2+</sup> and Cd<sup>2+</sup> (Sambongi et al. 1999), SDS, and quinine (Hilliard et al. 2004, 2005). Other amphid neurons play minor roles in either chemotaxis or avoidance (Bargmann and Horvitz 1991; Sambongi et al. 1999; Hilliard et al. 2002). The phasmid sensory neurons PHA and PHB appear to detect repulsive stimuli and coordinate avoidance responses by antagonizing ASH sensory neurons in the amphid (Hilliard et al. 2002). Recently, a set of sensory neurons, including URX, AQR, and PQR, have been shown to be involved in aerotaxis, or attraction to oxygen (Chang et al. 2006; Gray et al. 2004; Rogers et al. 2006). In addition, the BAG sensory neurons are involved in avoidance of  $CO_2$  (Hallem and Sternberg 2008) (Table 1.1). A few amphid neurons (ASI, ADF, ASJ, and ASG) play clear roles in dauer formation and maintenance, but make only minor contributions to behavioral chemosensory response (dauer is an alternative third larval stage, which is adopted under harsh conditions and starvation, is long-lived and resistant to environmental stress; see Figure 1.1, and reviewed in Fielenbach and Antebi 2008). IL2 labial neurons may also impact dauer recovery (Ouellet et al. 2008).

What determines if a particular chemosensory neuron drives attractive or repulsive behavior? Normally, AWA neurons drive response to the attractive odorant diacetyl (used as artificial butter odor in popcorn). When AWA neurons are killed with a laser microbeam, *C. elegans* are no

TABLE 1.1				
Chemosensory	Neurons	in	С.	elegans

Neuron(s)	Function	Compound(s) Sensed	Notes
ADF	Chemotaxis (minor)	cAMP, biotin, NaCl, lysine, NH <sub>4</sub> <sup>+</sup> (?), CH <sub>3</sub> COO <sup>-</sup> (?)	
ADL	Avoidance	1-Octanol	
ASE	Chemotaxis	ASEL: Na <sup>+</sup>	
		ASER: K <sup>+</sup> , Cl <sup>-</sup>	
		ASE (unknown): $NH_4^+(?)$ ,	
		CH <sub>3</sub> COO <sup>-</sup> (?)	
ASH	Avoidance	1-octanol, SDS, quinine, Cu <sup>2+</sup> , Cd <sup>2+</sup> , H <sup>+</sup>	
ASI	Chemotaxis (minor)	cAMP, biotin, NaCl, lysine, NH <sub>4</sub> <sup>+</sup> (?), CH <sub>3</sub> COO <sup>-</sup> (?)	
ASG	Chemotaxis (minor)	cAMP, biotin, NaCl, lysine, NH <sub>4</sub> <sup>+</sup> (?), CH <sub>3</sub> COO <sup>-</sup> (?)	
ASJ	Chemotaxis (minor)	Na <sup>+</sup>	
ASK	Chemotaxis (minor)	Attractant: lysine	
	Avoidance (minor)	Repellant: SDS	
AWA	Chemotaxis	Diacetyl, pyrazine, isoamyl alcohol (minor), 1,4,5- trimethylthiazole	Other unknown neurons sense pyrazine, diacetyl, and 1,4,5-trimethylthiazole
AWB	Avoidance	1-Octanol, 2-butanone	
AWC	Chemotaxis	AWC <sup>ON</sup> : butanone	
		AWC <sup>OFF</sup> : 2,3-pentanedione	
		AWC (both): benzaldehyde,	
		isoamyl alcohol	
		AWC (unknown): NH <sub>4</sub> <sup>+</sup> (?), 1,4,5-trimethylthiazole,	
		diacetyl (minor)	
SDQ, ALN, and/or PLN	Aerotaxis (minor)	O <sub>2</sub>	Nonamphid neurons; exposed to pseudocoelomic cavity
URX, AQR, PQR	Aerotaxis	$O_2$	-
BAG	Avoidance	$CO_2$	
PHA, PHB	Avoidance (antagonistic)	SDS	Phasmid neurons

*Note:* Neurons are amphid neurons unless otherwise stated. Only chemosensory functions are listed; note that some of these neurons have been shown to have other functions, including (but not limited to) regulation of lifespan, dauer formation, and mechanosensation. A question mark indicates that function has been inferred genetically with mutants that perturb neuron function and/or development, but has not been confirmed by laser ablation.

longer attracted to diacetyl. AWB neurons, on the other hand, normally drive avoidance responses to the volatile repellant 2-nonanone. When AWB neurons are killed, *C. elegans* no longer avoid 2-nonanone. The G-protein-coupled receptor (GPCR), ODR-10, is the olfactory receptor protein for diacetyl; ODR-10 is normally expressed in AWA neurons, and animals lacking ODR-10 are not attracted to diacetyl (see Section 1.2.1). When ODR-10 is heterologously expressed in AWB neurons, transgenic animals are repelled by diacetyl (Troemel et al. 1997). This suggests that in some cases, sensory neurons are developmentally hard-wired for attractive or repulsive behaviors and their synaptic targets determine the behavioral response.

A more recent study has shown that this strict labeled line paradigm may be less rigid than previously thought. AWC neurons (more specifically, AWC<sup>ON</sup> neurons; see Section 1.1.2.3), which

normally mediate attraction to the volatile chemical butanone, mediate avoidance to butanone in a *gcy-28* mutant background (Tsunozaki et al. 2008). *gcy-28* codes for a receptor-like guanylyl cyclase that may act through the diacyl glycerol kinase DGK-1 and the protein kinase C TTX-4/ PKC-1. This switch in behavioral encoding may result in a change in navigational strategy (see Section 1.4) rather than a change in neuronal wiring, as *gcy-28* function in adults is sufficient for normal butanone attraction.

#### 1.1.2.3 Asymmetrical Function in Anatomically Symmetric Pairs

Anatomically, amphid sensory neurons form bilaterally symmetric pairs. For example, ASEL and ASER are the left and right neurons in the ASE neuron pair, respectively. While most left-right pairs seem to be functionally identical, at least two pairs of sensory neurons, the ASE and AWC neuron pairs, are asymmetric in terms of their function.

The first asymmetry was discovered in the ASE neurons. The guanylyl cyclase gene, gcy-5, is exclusively expressed in ASER, whereas the related genes, gcy-6 and gcy-7, are only expressed in ASEL (Yu et al. 1997). Indeed, the ASEL and ASER neurons are also functionally distinct. ASEL neurons preferentially detect Na<sup>+</sup> ions, whereas the ASER neurons detect K<sup>+</sup> and Cl<sup>-</sup> ions (Pierce-Shimomura et al. 2001). A well-characterized regulatory cascade controls this difference in gene expression. Specification of the ASER cell fate, which appears to be the default state, requires the homeodomain protein CHE-1 (Uchida et al. 2003). The zinc finger transcription factors, LSY-2 (Johnston and Hobert 2005) and DIE-1 (Chang et al. 2004), are specifically expressed in ASEL and are required for the cell-specific expression of the microRNA (miRNA) lsy-6 in ASEL. Another miRNA, mi-273, functions in ASER to repress DIE-1 expression (Chang et al. 2004). The lsy-6 miRNA represses expression of the Nkx-type homeodomain protein COG-1 (Johnston and Hobert 2003). COG-1 acts together with UNC-37/Groucho to repress expression of ASEL-specific markers, such as gcy-7 and lim-6 (Chang et al. 2003). LIM-6, which is a homeodomain transcription factor, in turn represses expression of ASER-specific markers such as gcy-5. It is not clear what directly regulates ASEL-specific expression of LSY-2 and DIE-1, but ASE left-right asymmetry is specified early in embryogenesis in a LIN-12 Notch-dependent pathway that acts through the T-box transcription factors TBX-37 and TBX-38 (Poole and Hobert 2006). A substantial number of mutants defective in ASE left-right asymmetry have recently been isolated in a large-scale genetic screen (Sarin et al. 2007), indicating a wealth of knowledge waiting to be discovered.

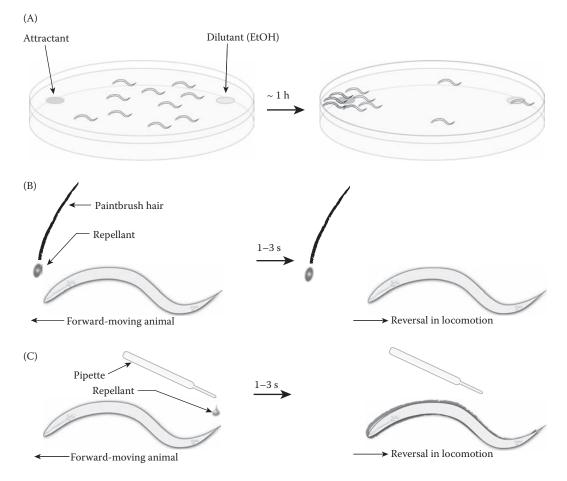
AWC neurons also show left-right asymmetry. However, unlike the ASE neurons, wherein the left-right cell fate is developmentally hard-wired, an AWC neuron can randomly adapt one of two fates, with the other AWC neuron in the pair developing the other fate. AWC cell fate decisions are marked by expression of *str-2*, which codes for a putative olfactory receptor (see below): one AWC neuron expresses str-2 (the "str-2 on" or AWCON fate) and the other does not (the "str-2 off" or AWC<sup>OFF</sup> fate) (Troemel et al. 1999). The AWC<sup>ON</sup> neuron detects the volatile attractant butanone, whereas the AWC<sup>OFF</sup> neuron detects the volatile attractant 2,3-pentanedione: both AWC neurons detect another attractant, benzaldehyde (Wes and Bargmann 2001). Proper AWC leftright specification requires synaptic contact between the neurites of the left-right AWC neurons (Troemel et al. 1999). The voltage-gated Ca<sup>2+</sup> channel subunits UNC-2 and UNC-36 are required for proper left-right specification. Influx of Ca<sup>2+</sup> probably activates the Ca<sup>2+</sup> modulated protein kinase UNC-43, which then activates NSY-1/ASK1, a MAP kinase-kinase (Sagasti et al. 2001). A MAP kinase-signaling cascade is likely to inhibit str-2 expression, thereby promoting the AWC<sup>OFF</sup> cell fate. NSY-4 and NSY-5 appear to function in parallel to induce the AWC<sup>ON</sup> fate. NSY-4 is a claudin superfamily protein related to the  $\gamma$ -subunits of voltage-gated Ca<sup>2+</sup> channels (VanHoven et al. 2006). NSY-5/INX-19 is an innexin protein that forms gap junction channels. NSY-5 appears to act cell autonomously in AWC neurons to promote the AWC<sup>ON</sup> fate during development, but it also appears to function in other neurons (ASH and AFD) as well (Chuang et al. 2007).

# 1.1.3 CHEMOSENSORY ASSAYS IN CAENORHABDITIS ELEGANS

There are many variations of *C. elegans* chemosensory assays that have been employed by researchers, but they can be grouped into a few basic techniques (Figure 1.4).

# 1.1.3.1 Chemotaxis Assays (Attractants)

Quantitative measurement of attraction to chemicals is reported as a chemotaxis index (CI), which reflects the response of a population of animals (Bargmann et al. 1993). A large, round Petri dish containing normal *C. elegans* culture media is used (NGM agar); no bacterial lawn (i.e., food) is



**FIGURE 1.4** *C. elegans* chemosensory assays. (A) The chemotaxis assay. Animals are placed on a large Petri dish, on which a spot of a volatile attractant and a dilutant have been placed on opposite sides of the plate (ethanol is usually used as a dilutant because *C. elegans* are mostly indifferent to it). A drop of sodium azide, which paralyzes the animals, is also placed with the odorant and dilutant. Animals are placed in the middle of the plate and are allowed to crawl around freely for about 1 h. The formula for calculating the CI is described in the text; in this example, CI=0.6. (B) The "smell-on-a-stick" assay. A paintbrush hair is dipped in a volatile chemical repellant and presented to the anterior tip of a forward-moving animal, where the amphid pore is located (see Figure 1.2). The hair is not allowed to touch the animal. Animals typically respond rapidly by reversing direction, usually within seconds. (C) The drop assay. A drop of soluble chemical repellant is applied to the tail of the animal. Capillary action carries the solution to the amphid pore at the tip of the nose. Animals typically respond rapidly by reversing direction, usually within seconds.

present. For volatile attractants, the chemical of interest (usually diluted in ethanol) is pipetted onto a spot on one side of the dish; a drop of the dilutant is added to the opposite side as a control. An anesthetic (usually sodium azide) is also added at each spot. Animals are placed between the two drops for the assay, and those that reach one of the spots are anesthetized and thus immobilized. After ~1 h, the number of animals that have accumulated at each spot is determined. The CI is then calculated as:

$$CI_{volatile} = \frac{(No. \text{ worms at attractant}) - (No. \text{ worms at control})}{Total no. \text{ worms}}$$

Thus, the closer the CI is to 1.0, the stronger the attraction to the odorant; a CI close to zero means that the odorant is neutral to animals; and a negative CI suggests that animals are repelled by the odorant.

Originally, the CI was actually designed for soluble attractants. Agar plugs containing high concentrations of an attractant are placed snugly into holes in an assay plate and gradients are allowed to develop overnight via passive dilution (Bargmann and Horvitz 1991); assays are otherwise similar. In a variation of this assay, a round Petri dish is divided into four quadrants (Wicks et al. 2000). Two nonadjacent quadrants are filled with NGM containing the soluble attractant, whereas the remaining quadrants lack the attractant. Animals are allowed to disperse from the center of the dish during the assay. The CI is then calculated as above.

#### 1.1.3.2 Avoidance Assays for Repellants

A variation of the chemotaxis assay can be used to measures active avoidance of volatile compounds (Troemel et al. 1997). However, more recent studies on volatile chemical repellants (Chao et al. 2004; Ferkey et al. 2007; Fukuto et al. 2004; Wragg et al. 2007) utilize the more rapid "smellon-a-stick" assay (Troemel et al. 1995). A thin paintbrush hair taped to a Pasteur pipette is dipped into a volatile odorant (e.g., octanol or nonanone) and placed in front of an animal that is crawling forward (the hair is not allowed to contact the animal). A wild type *C. elegans* rapidly initiates backward locomotion, usually within seconds. Avoidance is reported as average time required to initiate a reversal. It is likely that the circuitry and genetic requirements for these two assays (populations vs individual animals) may not be absolutely identical. The advantage of the individual assay over the plate assay is that it measures an immediate response and that individual animals are easier to assay.

For soluble repellants, a plate-based assay can be used wherein a barrier of the repellant is "painted" on the dish to form an enclosed border. Animals are placed inside the border and the avoidance index is measured as the number of *C. elegans* that are retained in the border divided by the total number of animals (Wicks et al. 2000). An interesting variation of this assay tested the navigational abilities of *C. elegans*. A Cu<sup>2+</sup> maze was painted onto the agar surface, revealing navigational defects in animals lacking NMR-1, a *C. elegans* homolog of an NMDA-type glutamate receptor subunit (Brockie et al. 2001). Another assay used for soluble repellants is the drop assay (Hilliard et al. 2002, 2004). In this assay, a capillary micropipette is used place a drop of repellant on the tail of a forward-moving animal. Capillary action causes the repellant to move to the animal's nose, and a reversal is initiated. The behavior is then scored similar to the smell-on-a-stick assay. The role of the phasmid neurons in chemosensory response was revealed using this assay (Hilliard et al. 2002).

# 1.1.3.3 Neuroimaging Approaches

Recently, techniques have been developed for directly imaging neural activity in *C. elegans* neurons upon mechanical or chemical stimulation. In *C. elegans* (as well as the parasitic nematode

Ascaris suum),  $Ca^{2+}$  is thought to be the major cation that carries depolarizing currents (Davis and Stretton 1989; Goodman et al. 1998). Imaging neural activity takes advantage of the genetically encoded Ca2+ indicator, cameleon (Miyawaki et al. 1997). Cameleon consists of CFP (the cyan variant of GFP) fused to YFP (the yellow variant of GFP), linked by calmodulin and the M13 calmodulin-binding domain. When Ca2+ is present at sufficient levels, calmodulin wraps around the M13 domain, thereby closely juxtaposing CFP and YFP. When cameleon is exposed to CFP excitation wavelengths, this conformational change causes fluorescence resonance energy transfer (FRET); YFP excitation only occurs via nearby photonic energy release from CFP emission. Both CFP and YFP signal are acquired in real-time using a beam splitter, and Ca<sup>2+</sup> flux is measured as a change in CFP/YFP ratio. Cameleon can be expressed in specific neurons using well-characterized promoters with defined expression patterns. Due the small size of C. elegans neurons, most cameleon measurements are made with animals that have been immobilized in some way. Early Ca<sup>2+</sup> imaging experiments utilized veterinarian's glue (Kerr et al. 2000); subsequent advances in the field now utilize microfluidic chambers that either immobilize animals using a custom microfabricated elastomer trap (Chronis et al. 2007) or that allow free movement in an environment that mimics soil and tracks freely moving animals at high magnification using computer-controlled stages (Lockery et al. 2008). Similar types of in vivo  $Ca^{2+}$  flux measurements can be performed in C. elegans (e.g., Tsunozaki et al. 2008; Chalasani et al. 2007) using G-CaMP, a nonratiometric Ca2+ indicator based on a circularly permutated GFP (Nakai et al. 2001). Ca<sup>2+</sup> imaging is typically performed on cell bodies, but in some cases, imaging of the neurites can also be performed (Chalasani et al. 2007; Clark et al. 2006).

# **1.2 SIGNAL TRANSDUCTION**

The literature on *C. elegans* chemosensory signal transduction is extensive, and the nomenclature can be confusing. Table 1.2 lists *C. elegans* protein/gene names and a concise description of their function and vertebrate counterparts, where applicable; and Figure 1.5 summarizes the relevant signaling pathways.

#### **1.2.1** SEVEN TRANSMEMBRANE DOMAIN OLFACTORY RECEPTORS

The C. elegans genome contains a dizzying array of genes that encode seven transmembrane serpentine receptors; most of these are presumed to encode olfactory receptors that probably couple to heterotrimeric G-proteins. Based on phylogenetic analyses, these receptors can be roughly classified into four superfamilies: str, sra, srg, and srw. Collectively, family members compromise ~1300 receptor genes and ~400 pseudogenes (Robertson and Thomas 2006). Expression analysis using promoter GFP fusion reporters of a representative subset of these genes suggests that these receptor genes are expressed in chemosensory neurons (Troemel et al. 1995). Neuron-specific gene expression profiling also indicated expression of a subset of putative olfactory receptors in AWB chemosensory neurons (Colosimo et al. 2004), which is consistent with a chemosensory function for these receptors. Interestingly, there is very little functional information on these odorant receptors. ODR-10, a member of the str superfamily, was identified in a classical genetic screen for animals defective for chemotaxis toward the volatile attractant diacetyl, and remains the only functionally defined C. elegans olfactory receptor with a defined ligand (Sengupta et al. 1996) (Figure 1.5B). It is noteworthy that after the seminal work of Buck and Axel (1991) in discovering olfactory receptor gene families in mammalian olfactory neurons, ODR-10 was the first seven transmembrane receptor in any organism to be functionally characterized as an olfactory receptor. SRA-13, a member of the sra superfamily, acts antagonistically with C. elegans Ras/MAPK signaling to negatively regulate chemotaxis to diacetyl and another attractant, isoamyl alcohol. However, it is unknown if SRA-13 codes for a bona fide chemoreceptor or has some kind of constitutive regulatory

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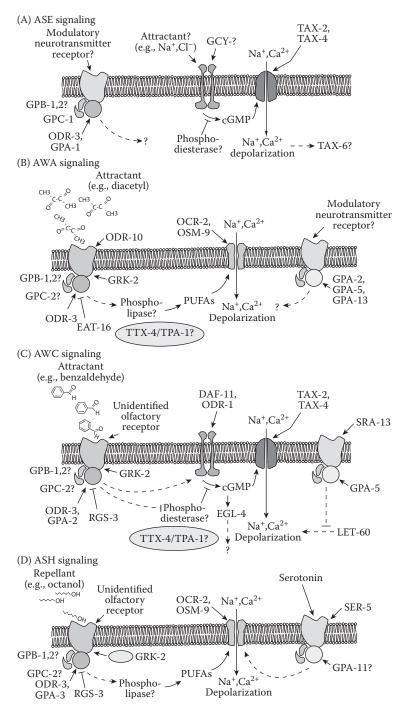
# TABLE 1.2List of Proteins and Genes Involved in C. elegans Chemosensation

List of Froteins and Genes inforced in et cregans enemotiensation				
	C. elegans			
General	Protein/Gene			
Description	Name	Description	Notes	
G-proteins and related proteins	GPA-1–GPA-16, ODR-3	Heterotrimeric G-protein α subunits	Chemosensory function of some is inferred by expression pattern; some function in modulation and/or regulation of olfaction	
	GPB-1, GPB-2	Heterotrimeric G-protein β subunits	Widely expressed in neuronal and non-neuronal tissues	
	GPC-1	Heterotrimeric G-protein γ subunit	Involved in salt adaptation; probably not involved in primary olfactory signaling	
	GPC-2	Heterotrimeric G-protein γ subunit	Probably involved in primary olfactory signaling; not functionally confirmed	
	EAT-16	Regulator of G-protein signaling (RGS protein)	Regulates AWA neurons	
	RGS-3	Regulator of G-protein signaling (RGS protein)	Regulates AWC and ASH neurons	
	LET-60	Ras	Required for AWC-mediated chemotaxis	
G-protein-	STR-xx, SRA-xx,	Orphan receptors (over 1000)	Inferred to be olfactory receptors based	
coupled	SRG-xx, SRW-xx		on expression patterns	
receptors (GPCRs)	SRA-13	Orphan receptor	Negatively regulates LET-60 in AWC neurons	
	STR-2	Orphan receptor	Marks AWCON/OFF cell fates	
	ODR-10	Diacetyl receptor	Only <i>C. elegans</i> nonorphan olfactory receptor; member of STR receptor superfamily	
	NPR-1	Neuropeptide receptor	Modulates aerotaxis and feeding behavior	
	SER-5	Serotonin receptor	Modulates octanol avoidance	
	F14D12.6	Octopamine receptor	Regulates octanol avoidance	
	TYRA-3	Tyramine receptor	Regulates octanol avoidance	
Kinases	GRK-2	GPCR kinase	Essential for chemotaxis and avoidance behaviors	
	TTX-4/PKC-1, TPA-1	Ca <sup>2+</sup> -independent protein kinase C	Required for AWA and AWC function; also required for AWC odor preference	
	EGL-4	cGMP-dependent kinase	Required for olfactory adaptation; in <i>Pristionchus</i> nematodes required for host pheromone detection	
	DGK-1	Diacyl glycerol kinase	Regulates AWC odor preference	
	UNC-43	Ca2+ modulated kinase	AWC <sup>ON/OFF</sup> cell fate specification	
	NSY-1	ASK1, a MAP kinase-kinase- kinase	AWC <sup>ON/OFF</sup> cell fate specification	
Guanylyl cyclases	DAF-11, ODR-1	Receptor-like transmembrane guanylyl cyclases	Required for AWC signaling; also required for ASE signaling but noncell autonomously	
	GCY-35, GCY-36	Soluble guanylyl cyclases	O <sub>2</sub> receptors, required for aerotaxis	
	GCY-28	Receptor-like transmembrane guanylyl cyclase	Regulates AWC odor preference	

General	C. elegans Protein/Gene		
Description	Name	Description	Notes
Channels	TAX-2, TAX-4	cGMP-gated channels; probably form heterodimers	Required for ASE and AWC signaling; also required for aerotaxis and CO <sub>2</sub> avoidance
	OCR-2, OSM-11	TRPV channels	Required for AWA and ASH signaling; activated by PUFAs
	EGL-19	L-type voltage-gated Ca <sup>2+</sup> channel α1 subunit	Presumably required for propagation of membrane depolarization in neurites
	UNC-2	L-type voltage-gated Ca <sup>2+</sup> channel α1 subunit	AWC <sup>ON/OFF</sup> cell fate specification
	UNC-36	L-type voltage-gated Ca <sup>2+</sup> channel, $\alpha 2/\delta$ subunit	$AWC^{\text{ON/OFF}}$ cell fate specification
	GLR-1	AMPA/kainate-like glutamate receptor	Expressed in command interneurons, AIB interneurons
	NMR-1	NMDA-like glutamate receptor	Expressed in command interneurons
	GLC-3	Glutamate-gated Cl <sup>-</sup> channel	Expressed in AIY interneurons
Signaling	FLP-18, FLP-21	FMRF amide-like neuropeptides	Ligands for NPR-1
ligands	DAF-2	Insulin-like peptide hormone	Suppresses CO <sub>2</sub> avoidance
	DAF-7	TGFβ homolog	Suppresses $CO_2$ avoidance
Transcription	CHE-1	Homeodomain protein	ASER cell fate specification
factors	LSY-2, DIE-1	Zn-finger proteins	ASEL cell fate specification
	COG-1	Nkx-type homeodomain protein	ASER cell fate specification
	UNC-37	Groucho homolog; transcriptional corepressor	ASER cell fate specification
	LIM-6	Homeodomain protein	ASEL cell fate specification
	TBX-37, TBX-38	T-box transcription factors	ASEL/R cell fate specification
	HIF-1	Hypoxia-induced factor	Suppresses CO <sub>2</sub> avoidance defects in <i>egl-9</i> mutants
	NHR-49	Orphan nuclear hormone receptor	Required for CO <sub>2</sub> avoidance
Other	QUI-1	Novel WD40 repeat protein	Required for quinine avoidance
	OSM-10	Novel protein	Required for osmo avoidance
	EGL-9	Prolyl hydroxylase	Required for $CO_2$ avoidance
	lsy-6	MicroRNA	ASEL cell fate specification
	mi-273	MicroRNA	ASER cell fate specification
	LIN-12	Notch receptor homolog	ASEL/R cell fate specification
	NSY-4	Claudin superfamily protein	AWC <sup>ON/OFF</sup> cell fate specification
	NSY-5/INX-19	Innexin	AWC <sup>ON/OFF</sup> cell fate specification
	UNC-101	Clathrin adapter protein	Olfactory receptor trafficking
	ODR-4	Novel conserved protein	Olfactory receptor trafficking
	odr-8	Uncloned gene	Olfactory receptor trafficking
	ARR-1	Arrestin	Olfactory adaptation
	CAT-2	Tyrosine hydroxylase	Dopamine biosynthesis

#### TABLE 1.2 Continued

*Note:* This list is not meant to be comprehensive; only proteins and genes discussed in this chapter are listed. For GPCRs, "xx" indicates a number; there are approximately 1000 putative olfactory receptors encoded by the *C. elegans* genome.



**FIGURE 1.5** Signal transduction pathways of major *C. elegans* chemosensory neurons. Protein names followed by a question mark indicate that the position of that protein in the pathway is ambiguous. Dotted lines between signaling components indicates that there are an indeterminate number of unidentified molecules between the known components. Stand-alone question marks indicate a signaling pathway that exerts a modulatory and/or regulatory effect on chemosensory signaling, but with an unknown mechanism. For a list of functions of the signaling proteins shown in this figure, see Table 1.2. PUFAs: polyunsaturated fatty acids. (A) Signaling in soluble attractant-sensing ASE neurons; (B) and (C) signaling in volatile attractant-sensing AWA and AWC neurons, respectively; (D) signaling in volatile attractant-sensing ASH neurons.

function (Battu et al. 2003). At least one genome-wide RNA interference (RNAi) screen unrelated to chemotaxis identified several putative chemoreceptor genes that are involved in fat metabolism (Ashrafi et al. 2003), suggesting that these receptor proteins may have diverse functions that are currently unexplored.

In contrast with the large number of olfactory receptor genes, *C. elegans* has a limited repertoire of chemosensory neurons. Thus, multiple receptors are expressed in each sensory neuron. This sort of anatomical limitation can be thought of as a more extreme example of the organization of olfactory receptors in *Drosophila*, where some olfactory neurons express two to three different receptors (reviewed in Fiala 2007; see also Chapter 2). This is in contrast to the organization of the vertebrate olfactory system, wherein a single olfactory receptor gene is expressed per sensory neuron (Chess et al. 1994), and odor perception is interpreted when a combination of different olfactory receptors (and therefore different sensory neurons) are activated in response to a single odorant (Malnic et al. 1999, see also Chapter 7).

The mechanism of olfactory receptor trafficking and insertion into the membrane is poorly understood (Bush and Hall 2008). Olfactory receptor proteins are actively trafficked to the sensory cilia in a process that depends on *unc-101*, which codes for a clathrin adapter protein. There also appear to be trafficking pathways for olfactory receptors that act semiredundantly with *unc-101* (Dwyer et al. 2001). Olfactory receptors (and GPCRs, in general) do not target the plasma membrane efficiently when expressed in heterologous cells. ODR-4 is a novel protein that is required for proper localization of the olfactory receptor ODR-10 to the sensory cilia in *C. elegans* (Dwyer et al. 1998). When ODR-4 is expressed in mammalian cell lines, it facilitates proper trafficking of at least one rat olfactory receptor (Gimelbrant et al. 2001). ODR-4 is apparently conserved in vertebrates (Lehman et al. 2005), although no functional studies on this protein have been done other than in *C. elegans. odr-8* is another *C. elegans* gene required for proper trafficking of ODR-10, but it has not yet been cloned (Dwyer et al. 1998).

GPCR kinases (GRKs) impact neuron response in C. elegans differently than expected, based on vertebrate studies. In vertebrates, olfactory signal transduction is negatively regulated by phosphorylation of olfactory receptors by GRKs and  $\beta$ -arrestin (Dawson et al. 1993). Odorant stimulation of wild-type olfactory epithelia in mice leads to rapid desensitization of cAMP formation, but this desensitization is absent in GRK-3 knockout mice (Peppel et al. 1997). In C. elegans, loss of function of the GRK homolog grk-2 leads to loss of behavioral response to attractive and repulsive odorants. The loss of behavioral response could not be attributed to a loss of desensitization; rather, loss of grk-2 function presumably leads to decreased G-protein signaling, likely via a feedback mechanism. Loss of function in the C. elegans  $\beta$ -arrestin gene arr-1 does not overtly affect chemotaxis (Fukuto et al. 2004). However, loss of function in arr-1 affects adaptation and recovery from adaptation to odorants (Palmitessa et al. 2005). These results suggest that GRK signaling (specifically GRK-2) in C. elegans might positively regulate chemosensation in a fashion that is distinct from GRK regulation of odorant receptors in mammals (Figure 1.5B through D), but that C. elegans ARR-1 functions more analogously to mammalian arrestin proteins. The C. elegans genome contains two GRK homologs, and GRK-1 has not yet been characterized. One possibility is that GRK-1, and not GRK-2, acts in conjunction with ARR-1 to regulate adaptation of chemosensation. Alternatively, GRK-2 may play more than one role in chemosensory neurons.

#### **1.2.2** HETEROTRIMERIC G-PROTEINS

*C. elegans* olfactory receptors probably couple to heterotrimeric G-proteins. The *C. elegans* genome contains 21 genes encoding heterotrimeric G $\alpha$  subunits (Jansen et al. 1999). *gsa-1, goa-1, egl-30*, and *gpa-12* code for homologs of mammalian G<sub>s</sub>, G<sub>i/o</sub>, G<sub>q</sub>, and G<sub>12/13</sub>  $\alpha$  subunits, respectively. These conserved genes are expressed in a variety of cell types including neurons. (Note that *gpa-12* expression is somewhat more limited.) GOA-1 and EGL-30 are required for olfactory adaptation

(see below), but not olfaction itself (Matsuki et al. 2006). There is no obvious C. elegans homolog for  $G_{olf}$ , the G $\alpha$  subunit used for signal transduction in mammalian olfactory neurons. The remaining C. elegans G $\alpha$ -coding genes (the gpa genes and odr-3) are G<sub>i</sub>-like but unique to C. elegans, and 14 of these are expressed almost exclusively in chemosensory neurons. Immunohistochemistry showed that some  $G\alpha$  subunits are expressed in sensory cilia (Lans et al. 2004; Roayaie et al. 1998), suggesting that they might play a direct role in chemosensation. The first identified noncanonical  $G_i$ -like  $G\alpha$  subunit, ODR-3, was identified in a genetic screen for mutants defective for chemotaxis toward benzaldehyde, an attractive odorant detected by the AWC sensory neurons (Roayaie et al. 1998; Bargmann et al. 1993). The different  $G\alpha$  subunits have distinct and complex contributions to olfactory signal transduction in specific neurons. For instance, odr-3 loss-of-function animals are strongly defective for chemotaxis to the attractants diacetyl, pyrazine (detected by AWA neurons), and isoamyl alcohol (detected by AWC neurons) (Figure 1.5). However, detection of butanone (also detected by AWC neurons) requires the redundant function of GPA-2 and ODR-3 (Roayaie et al. 1998) (Figure 1.5C). ODR-3 and GPA-3 both function in ASH sensory neurons to facilitate avoidance responses (Figure 1.5D). odr-3 mutant animals are defective for response to the chemical repellant octanol, but not quinine (Fukuto et al. 2004; Hilliard et al. 2004). gpa-3 mutant animals are weakly defective for response to octanol (M.Y. Chao and A.C. Hart; unpublished observations) and quinine, but odr-3;gpa-3 double mutants are strongly defective for quinine response (Hilliard et al. 2004). One interpretation of these data is that some receptors may couple only to specific  $G\alpha$ subunits, whereas other receptors may be more promiscuous. Other GPA proteins may have regulatory functions. For instance, GPA-11 modulates the activity of ASH neurons, depending on the presence or absence of food (Chao et al. 2004) (Figure 1.5D). GPA-2, GPA-5, and GPA-13 also have regulatory roles in AWA and AWC neurons (Lans et al. 2004) (Figure 1.5B and C). One possibility is that these GPA proteins may couple to biogenic amine neurotransmitter receptors, which may have modulatory rather than excitatory activity. Serotonin, dopamine, octopamine, and tyramine have all been shown to modulate C. elegans chemosensory response (Chao et al. 2004; Ferkey et al. 2007; Wragg et al. 2007) (see Section 1.3.2).

*C. elegans* has two G $\beta$  (GPB-1 and GPB-2) and two G $\gamma$  (GPC-1 and GPC-2) subunits (Jansen et al. 1999). GPB-1, GPB-2, and GPC-2 are widely expressed in many tissues, while GPC-1 expression is restricted to a limited set of sensory neurons. Due to the limited number of G $\beta$  and G $\gamma$  proteins, the various G $\alpha$  proteins probably share these G $\beta$  and G $\gamma$  proteins in limited combinations to form heterotrimers. GPB-1 is required for ASH neuron-mediated avoidance responses (Esposito et al. 2007). GPB-2 and GPC-1, while not directly required for chemosensation, are required for adaptation to NaCl, a type of chemosensory learning (Matsuki et al. 2006; Jansen et al. 2002) (Figure 1.5A). This suggests that GPC-2 may be the G $\gamma$  subunit that participates in general signal transduction in sensory neurons, although GPC-2 has not yet been functionally characterized in sensory neurons.

Regulators of G-protein signaling (RGS) proteins negatively regulate G-protein signaling by promoting GTP hydrolysis (Hollinger and Hepler 2002; Ross and Wilkie 2000). They have been shown to regulate chemosensory signaling in at least two cases. First, EAT-16 negatively regulates chemosensory signaling in AWA sensory neurons (Figure 1.5B). Loss-of-function mutations in *eat-16* suppress chemosensory behavioral defects in *grk-2* mutants. However, only defects for which the normal behavioral response is mediated by AWA neurons are suppressed; *grk-2* behavioral defects that are caused by impaired function in AWC neurons are not suppressed (Fukuto et al. 2004). Second, RGS-3 negatively regulates signaling in several chemosensory neurons, including AWC and ASH neurons (Ferkey et al. 2007) (Figure 1.5C and D). Interestingly, loss-of-function mutations in *rgs-3* resulted in defects in behavioral responses to strong stimuli but not weak stimuli, suggesting that one role of RGS signaling is gain control. Excessive signaling in sensory neurons caused by loss of *rgs-3* does not lead to a corresponding increase in behavioral output (Ferkey et al. 2007), suggesting that signal gain must fall within an optimal range for normal behavioral response.

## 1.2.3 DOWNSTREAM EFFECTORS OF CHEMOSENSORY SIGNALING

Different sensory neurons in *C. elegans* are specialized for specific sensory modalities. In terms of chemosensation, the major players can be classified into five groups: ASE neurons, which detect soluble attractants; AWA and AWC, which detect volatile attractants; ASH, ADL, and AWB, which detect volatile (and some soluble) repellants; ASI, ADF, and ASJ, which are involved in dauer formation and play minor roles in soluble attractants (dauer formation is a developmental phenomenon and is not explored further herein); and the sensory neurons involved in detection of  $O_2$  and  $CO_2$ . Each class utilizes different signaling mechanisms, although there is some overlap in the signaling molecules involved (Figure 1.5). Thus, our discussion will be organized on the basis of sensory modalities.

#### 1.2.3.1 Soluble Attractants: Chemotaxis toward NaCl and ASE Neurons

*C. elegans* is attracted to a variety of soluble chemicals, including Na<sup>+</sup>, Cl<sup>-</sup>, biotin, cAMP, lysine, and serotonin (Bargmann and Horvitz 1991). The best-studied soluble attractant for *C. elegans* is NaCl. Low concentrations of NaCl (0.1–200 mM) attract *C. elegans*, and this is mostly mediated by ASE neurons, with minor contributions from ADF, ASG, and ASI neurons (Bargmann and Horvitz 1991). High concentrations of NaCl (>200 mM) actually repel *C. elegans*, but this is probably due to a general osmotic avoidance mechanism involving the ASH neurons rather than a chemosensory avoidance response (Hukema et al. 2006).

The receptor(s) that mediate NaCl attraction are unknown. At least some seven transmembrane receptors of the putative chemoreceptor superfamily are expressed in ASE neurons, suggesting that detection of some soluble attractants is mediated by G-protein signaling (Etchberger et al. 2007). However, detection of NaCl may not involve G-protein signaling. When the entire complement of mutant strains lacking G $\alpha$  subunits expressed in ASE neurons were tested, none were defective for chemotaxis toward NaCl (Hukema et al. 2006). G-protein signaling does have important cell autonomous and noncell autonomous roles in behavioral plasticity related to chemotaxis to NaCl (Hukema et al. 2002).

NaCl attraction requires cGMP signaling. tax-2 and tax-4, which code for subunits of a cGMPgated channel, are required for attraction to NaCl (Coburn and Bargmann 1996; Komatsu et al. 1996). Vertebrate cyclic nucleotide-gated channels involved in sensory transduction, such as those in rod cells and olfactory neurons, function as heterotetramers (reviewed in Pifferi et al. 2006; see also Chapter 8), and based on in vitro evidence, TAX-2 and TAX-4 probably multimerize to form a heteromeric active channel (Komatsu et al. 1999). The source of cGMP is unknown. The receptorlike transmembrane guanylyl cyclases ODR-1 (L'Etoile and Bargmann 2000) and DAF-11 (Birnby et al. 2000) are required for NaCl attraction. However, neither ODR-1 nor DAF-11 are expressed in ASE neurons, suggesting that they act non-cell autonomously in NaCl attraction, and thus, are not the direct source of cGMP for TAX-2/TAX-4. The C. elegans genome encodes 34 guanylyl cyclases, 24 of which are transmembrane receptor-like proteins; of the transmembrane guanylyl cyclases, nine are expressed in ASE neurons, with some showing left-right asymmetry in their expression patterns (Ortiz et al. 2006). Since the left and right ASE neurons detect Na<sup>+</sup> and Cl<sup>-</sup>, respectively, one intriguing possibility is that transmembrane guanylyl cyclases function as Na<sup>+</sup> and Cl<sup>-</sup> receptors that mediate ASE NaCl attraction; currently, there is no functional evidence for this as of this writing. The C. elegans calcineurin protein TAX-6 is also required in sensory neurons (probably in ASE neurons) for chemotaxis toward NaCl, suggesting that Ca<sup>2+</sup> signaling is required (Kuhara et al. 2002). However, where TAX-6 functions in the signaling pathway is unknown (Figure 1.5A).

A recent study suggests that AWC neurons, which are typically assigned the function of sensing volatile odorants (see below), also sense  $NH_4^+$  ions in soluble form (Frøkjaer-Jensen et al. 2008). Ammonium acetate is an interesting odorant that is sensed both as a soluble and volatile compound, using a distributed combination of exposed (ASE and others) and nonexposed (AWA and/or AWC) ciliated sensory neurons (Frøkjaer-Jensen et al. 2008).

#### 1.2.3.2 Volatile Attractants: Chemotaxis Mediated by AWA and AWC Neurons

Attraction to volatile odorants is primarily mediated by the AWA and AWC sensory neurons, and is most likely a part of a general foraging strategy for *C. elegans* to locate bacteria and other food sources. In contrast to soluble attractants such as NaCl, wherein diffusion is limited by the presence of water, volatile odorants that diffuse through the air represent long-distance attractive cues. *C. elegans* responds to a variety of volatile organic compounds, including alcohols, ketones, aldehydes, esters, amines, sulfhydryls, acids, aromatic, and heterocyclic compounds (Bargmann et al. 1993). Using laser ablation, a subset of these odorants has been assigned as being detected by either AWA or AWC (Table 1.1). Signal transduction in AWA and AWC neurons both probably involve G-protein-coupled olfactory receptors, but there are important differences in downstream mechanisms.

In the AWA sensory neurons, olfactory receptors such as the diacetyl receptor ODR-10 probably couple to the  $G_i$ -like G $\alpha$  protein, ODR-3 (Roayaie et al. 1998). Activation of ODR-3 is likely to lead to the metabolic release of polyunsaturated fatty acids (PUFAs) from membrane phospholipids via an unidentified phospholipase activity (Kahn-Kirby et al. 2004). Mutants defective in poly unsaturated fatty acid (PUFA) biosynthesis (*fat* mutants) (Watts and Browse 2002) are defective for chemotaxis toward diacetyl and pyrazine (odorants sensed by AWA neurons), but not benzaldehyde (an odorant sensed by AWC neurons) (Kahn-Kirby et al. 2004). Gas chromatograph analysis of the fatty acid content in the *fat* mutants, together with behavioral analysis, suggested that arachidonic acid (AA) and eicopentaenoic acid (EPA) are the PUFAs most important for AWA neuron activity. AA and EPA are likely to directly activate the TRPV channels OSM-9 and OCR-2 (Colbert et al. 1997; Tobin et al. 2002) (Figure 1.5B).

In contrast, the major signal transduction pathway utilized by the AWC sensory neurons appears to be through the DAF-11/ODR-1 guanylyl cyclases and the TAX-2/TAX-4 cGMP-gated channels. *daf-11* and *odr-1* mutants are defective for AWC-mediated chemosensory responses (L'Etoile and Bargmann 2000; Vowels and Thomas 1994; Birnby et al. 2000). Unlike the ASE neurons, DAF-11 and ODR-1 are expressed in the AWC neurons, and are thus, most likely to be the cell autonomous source of cGMP (L'Etoile and Bargmann 2000). AWC neurons are probably glutamatergic (Chalasani et al. 2007) (see Section 1.4).

Several other signaling molecules have been implicated in AWC signaling, although their placement in the signaling pathway is unclear. Interestingly, the *C. elegans* Ras homolog, LET-60, is required for AWC-mediated chemotaxis, and probably acts downstream of TAX-2/TAX-4 (Hirotsu et al. 2000). The putative olfactory receptor, SRA-13, negatively regulates LET-60 Ras signaling via the G $\alpha$  protein GPA-5 (Battu et al. 2003). The Ca<sup>2+</sup>-independent protein kinase C (nPKC) TTX-4 is also required for AWC (and AWA) mediated behaviors, and the related nPKC TPA-1 acts semiredundantly with TTX-4 (Okochi et al. 2005). The cGMP-dependent kinase EGL-4 is required for olfactory adaptation in AWC neurons, but not for general olfaction (L'Etoile et al. 2002) (Figure 1.5C). Interestingly, the EGL-4 homolog in the related nematode, *Pristionchus pacificus*, is required for olfaction of insect pheromones (Hong et al. 2008a) (see Section 1.5.2).

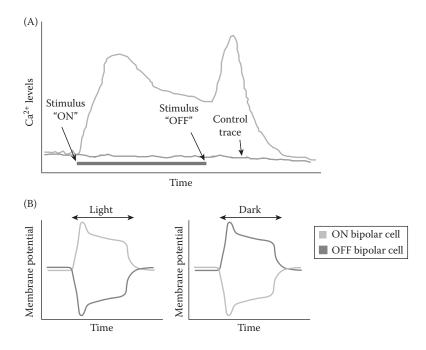
## 1.2.3.3 Chemical Repellants

Repellants are detected by at least three pairs of amphid neurons, ASH, ADL, and AWB, with minor contributions by other neurons. The AWB neurons require ODR-1 function for response to the repellant nonanone (L'Etoile and Bargmann 2000) and are involved in serrawettin avoidance (Pradel et al. 2007) (see 1.5.1) but otherwise are poorly characterized. The ASH neurons have been intensely studied. These polymodal neurons detect mechanical (Kaplan and Horvitz 1993) as well as soluble and volatile chemical stimuli. Here, we focus on the chemosensory role of ASH neurons. Laser ablation studies have shown that volatile repellents, such as the long-chain alcohol 1-octanol, are sensed by ASH neurons (ADL and AWB neurons also contribute to the response to volatile repellants; see below). ASH neurons also respond to soluble repellents, such as quinine, SDS, Cu<sup>2+</sup>, H<sup>+</sup>, and others (Dusenbery 1974; Sambongi et al. 1999; Hilliard et al. 2002, 2004). Using an analogy to bipolar cells in the vertebrate retinal nervous system (Yang 2004), ASH

neurons function as both ON-sensing and OFF-sensing cells Figure 1.6). Tonic increases in  $Ca^{2+}$  levels are detected in ASH neurons when ASH-activating stimuli are presented (Hilliard et al. 2005; Chronis et al. 2007), but transient  $Ca^{2+}$  spikes are also detected when those stimuli are removed (Chronis et al. 2007).

The ASH neurons synapse directly onto the command interneurons, unlike the AWA and AWC neurons that connect to the command interneurons through multiple layers of other interneurons (see Figure 1.2). This neuroanatomy reflects a difference in aversive vs attractive behavior; aversive responses are relatively rapid and occur on the order of several seconds, whereas attractive responses occur over a longer time span (30–60 min; see Figure 1.4). ASH signaling shares several components with AWA neurons. Odorants presumably are detected via GPCRs coupled to ODR-3, with GPA-3 playing a minor role. Similar to AWA neurons, activation of G-protein signaling presumably leads to the release of PUFAs from the plasma membrane, leading to activation of the ODR-9/OCR-2 TRPV channels (Kahn-Kirby et al. 2004) (Figure 1.5D). In addition to ODR-9, the L-type voltage-gated Ca<sup>2+</sup> channel, EGL-19, is also required for a transient increase in somatic Ca<sup>2+</sup> levels, as measured by the Ca<sup>2+</sup> indicator protein cameleon (Hilliard et al. 2005). The ASH neurons are probably glutamatergic (Lee et al. 1999), and chemosensory response requires the postsynaptic glutamate receptor GLR-1 (and probably others) expressed in the command interneurons (Chao et al. 2004; Mellem et al. 2002).

The ability of ASH neurons to respond to different sensory modalities requires the expression of modality-specific genes. For instance, OSM-10 is a novel cytoplasmic protein expressed in ASH neurons. Animals lacking OSM-10 fail to respond to osmotic stimuli, but still respond to volatile



**FIGURE 1.6**  $Ca^{2+}$  currents in ASH neurons after stimulation. (A) ASH neurons display a tonic increase in  $Ca^{2+}$  levels after stimulation with chemical repellants, such as quinine or  $Cu^{2+}$ . They also show a transient spike in  $Ca^{2+}$  levels after the stimulus is removed. (B) ASH neurons function similar to ON- and OFF-sensing bipolar cells in the vertebrate retina. Bipolar cells receive synaptic input from photoreceptors (not shown), which sense light. Bipolar ON cells depolarize when light is switched on, and hyperpolarize when the light is off. Conversely, bipolar OFF cells depolarize when light is switched off, and hyperpolarize when the light is on. ([A] Adapted from Chronis, N., M. Zimmer, and C. Bargmann. *Nat. Methods* 4, 727–31, 2007.)

and soluble repellants (Hart et al. 1999; Hilliard et al. 2005). Similarly, the novel WD-40 repeat protein QUI-1 (also expressed in ASH neurons) is required for avoidance of soluble repellant quinine (Hilliard et al. 2004) and the volatile repellant octanol (Fukuto 2004), but not for osmotic avoidance. The polymodal nature of ASH neurons appears to be an evolutionarily conserved trait among nematodes (Srinivasan et al. 2008).

#### 1.2.3.4 Oxygen and Carbon Dioxide

In soil or on bacterial lawns in laboratories, oxygen levels can vary widely over short distances. Physiologically, *C. elegans* can adapt to a wide range of oxygen concentrations. Metabolic rates remain relatively constant between 4 and 21 kPa of  $O_2$  (Van Voorhies and Ward 2000). At low (hypoxic) oxygen concentrations (0.25–1 kPa), *C. elegans* survive via a HIF-1-dependent hypoxic response pathway (Jiang et al. 2001). Alternatively, under anoxic conditions (<0.001 kPa), animals survive by entering a HIF-1 independent state of suspended animation (Nystul et al. 2003).

*C. elegans* respond behaviorally to changes in oxygen concentration. This behavior is termed aerotaxis. When animals are presented with a gradient of oxygen concentrations in specially designed chambers, they distribute between 4 and 12 kPa (Gray et al. 2004). *C. elegans* also expresses its preference for  $O_2$  concentration under standard laboratory culture conditions. Agar plates are typically seeded with a 100–200 µL drop of *E. coli* bacteria grown in liquid culture. As the liquid dries, the *E. coli* form a lawn that is slightly thicker at the edges. *C. elegans* of the laboratory wild-type reference strain (the N2 strain) typically disperse throughout the bacterial lawn to feed (solitary feeding), whereas certain wild isolates of *C. elegans* (e.g., the CB4856 strain from Hawaii; Wicks et al. 2001) tend to clump together, particularly at the edge of the bacterial lawn (social feeding). There is a measurably lower oxygen concentration in the thick edges of the bacterial lawn than the thinner regions in the middle of the lawn (Gray et al. 2004). The difference in oxygen preferences between different wild isolates is due to a single amino acid polymorphism in NPR-1, a G-protein-coupled FMRF amide-like neuropeptide receptor (de Bono and Bargmann 1998), and is discussed below.

Oxygen is sensed by the body cavity sensory neurons AQR, PQR, and/or URX, with contributions from the SDQ, ALN, and/or PLN neurons (Gray et al. 2004). Unlike the amphid neurons, AQR, PQR, and URX are exposed to the pseudocoelomic body fluids of the animal instead of the outside environment. Presumably, these neurons sense dissolved oxygen in the body cavity fluid. The  $O_2$  sensor proteins are the soluble heme-binding guanylyl cyclases (sGCs) GCY-35 and GCY-36 that bind  $O_2$ , and are unlike previously characterized soluble guanylyl cyclases that bind NO (Gray et al. 2004; Cheung et al. 2005; Chang et al. 2006). cGMP produced by these sGCs act via the TAX-2/TAX-4 cGMP-gated channels to activate the oxygen-sensing neurons (Chang et al. 2006). Aerotaxis toward the optimal  $O_2$  concentration also requires the TRPV channels ODR-9/OCR-2 acting in the nociceptive ASH and serotonergic ADF neurons (Chang et al. 2006; Rogers et al. 2006). Thus, aerotaxis appears to be mediated by a distributed network of neurons with different sensory modalities.

The NPR-1 neuropeptide receptor is expressed in AQR, PQR, URX, and other neurons (Coates and De Bono 2002), and is activated by the FMRF amide-like neuropeptides FLP-18 and FLP-21 (Rogers et al. 2003). Activation of NPR-1 probably antagonizes the activity of TAX-2/TAX-4 in AQR, PQR, and URX neurons (Coates and De Bono 2002). Thus,  $O_2$  levels sensed by the soluble guanylyl cyclases, GCY-35 and GCY-36, are likely to function as the primary signal to regulate social feeding, with other modes of sensory input modulating cGMP signaling via neuropeptides and NPR-1.

*C. elegans* also detects CO<sub>2</sub>. In contrast to O<sub>2</sub> sensing, wherein *C. elegans* migrate to an optimal O<sub>2</sub> concentration, *C. elegans* detects and actively avoids CO<sub>2</sub> concentrations greater than 0.5–1.0 pKa (Bretscher et al. 2008; Hallem and Sternberg 2008). This avoidance behavior can be measured using a concentration gradient similar to that used in assays for aerotaxis for O<sub>2</sub>, or

in acute puffs of CO<sub>2</sub> administered through a syringe. Atmospheric CO<sub>2</sub> levels (which is in the range of 300–400 ppm) are very low compared to atmospheric O<sub>2</sub>, but the natural microenvironment of *C. elegans* (bacteria-rich soil) can have very high local CO<sub>2</sub> concentrations (up to 10 kPa) (Sposito 2008). Thus, the detection threshold for CO<sub>2</sub> is well above atmospheric levels. This suggests that CO<sub>2</sub> avoidance is used by *C. elegans* to avoid potentially inhospitable environments in the soil. CO<sub>2</sub> avoidance requires the BAG sensory neurons (and probably other neurons), but does not require the AQR, PQR, and URX neurons required for aerotaxis toward O<sub>2</sub> (Bretscher et al. 2008). Some downstream signaling components of O<sub>2</sub> and CO<sub>2</sub> sensing are similar; both pathways require TAX-4 and are modulated by NPR-1. However, CO<sub>2</sub> detection does not require the RGS protein RGS-3 and Ca<sup>2+</sup> signaling via TAX-6/CNB-1 calcineurin (Hallem and Sternberg 2008).

### 1.3 MODULATORY PATHWAYS FOR CHEMOSENSORY BEHAVIORS

*C. elegans* modulates its behavioral responses to chemosensory stimuli, presumably based on a summation of different sources of sensory information. Modulation by the presence or absence of food is perhaps the best-studied phenomenon. Signaling via several modulatory neurotransmitters has been linked to changes in food availability. Among these, the best studied is the role of serotonin.

#### 1.3.1 FOOD AND SEROTONIN

#### 1.3.1.1 Relationship Between Food and Serotonin Signaling

Food is an important modulator of many *C. elegans* behaviors, including locomotion (Sawin et al. 2000), pharyngeal pumping (Avery and Horvitz 1990), male mating (Loer and Kenyon 1993), egg laying (Hajdu-Cronin et al. 1999; Horvitz et al. 1982), and chemosensory response (see below). The standard laboratory food source for *C. elegans* is *E. coli* bacteria. Ironically, although the effect of food on *C. elegans* behavior has been well characterized, the precise chemical and/or physical cues by which *C. elegans* detect *E. coli* remain unclear. A mechanical component is probable, as animals will slow when entering a bacterial lawn or a viscous substance (Sawin et al. 2000). Genetic evidence suggests that the presence of food increases overall levels of the modulatory neurotransmitter serotonin (Avery and Horvitz 1990; Colbert and Bargmann 1997; Sze et al. 2000). At least for locomotion and chemosensation, serotonin probably acts humorally rather than synaptically, as laser ablation of single pairs of serotonergic neurons has little effect on locomotory behavior and exogenous serotonin can compensate for genetic deficiencies in serotonin production (Sawin et al. 2000; Chao et al. 2004).

#### 1.3.1.2 Serotonin and Modulation of Chemosensation

*C. elegans* exhibits adaptation to attractive chemosensory stimuli. Briefly, if *C. elegans* are preexposed to benzaldehyde (an attractive odorant sensed by AWC neurons), their acute response to benzaldehyde is diminished compared to naive *C. elegans* that were not pre-exposed. When *C. elegans* are first deprived of food, adaptation to benzaldehyde is enhanced. Exogenous serotonin restores normal adaptation response, consistent with serotonin as the endogenous signal mediating olfactory adaptation (Colbert and Bargmann 1997).

Serotonin also has effects on the neural circuitry that mediates the avoidance response to the volatile chemical repellant, octanol. This effect is twofold. First, serotonin acts directly on the ASH sensory neurons to modulate response to octanol (Chao et al. 2004; Hilliard et al. 2005). Exogenous serotonin can directly potentiate Ca<sup>2+</sup> influx in ASH neurons (Hilliard et al. 2005). Serotonin probably modulates this response via at least three serotonin receptors: the GPCR SER-5, which is expressed in ASH neurons (Harris and Kommuniecki, Personal Communication); the serotonin-gated chloride channel

MOD-1 (Ranganathan et al. 2000), which potentially functions in AIY and/or AIB interneurons; and the GPCR SER-1, which potentially acts in RIA interneurons (Harris et al. 2009). The G $\alpha$  protein, GPA-11, which is also expressed in ASH neurons, is also required for serotonin modulation (Chao et al. 2004), although SER-5 may not directly couple to GPA-11 (G. Harris and R. Komuniecki, personal communication). Second, serotonin alters the neural circuitry used to detect octanol. When serotonin levels are high, C. elegans primarily utilize ASH neurons to sense octanol; when serotonin levels are decreased (caused by mild starvation, for instance), the ADL and AWB neurons are also recruited to sense octanol (Chao et al. 2004). In this second pathway, it is unclear whether serotonin acts presynaptically in sensory neurons or postsynaptically in interneurons (or elsewhere). Recruitment of ADL and AWB does not require GPA-11, but does require the glutamate receptor GLR-1, which is expressed in the command interneurons immediately postsynaptic to the sensory neurons. This suggests that serotonin may act on the command interneurons, but further studies are needed to clarify the mechanism of this switch in circuitry. Since the life cycle of C. elegans is so short and the developmental program of the organism is essentially invariant, plasticity at the level of neural circuitry may be preferred over de novo synaptogenesis. Clearly, this plasticity is exerted at multiple levels of the neural circuit, and reflects the complexity of the modulatory input into a very simple behavioral response.

#### 1.3.1.3 Food and Modulation of CO<sub>2</sub> Avoidance

Food also modulates C. elegans avoidance of  $CO_2$ , although signaling pathways other than those used for serotonin may be involved. Avoidance of  $CO_2$  is partially suppressed by starvation, which activates the DAF-2 insulin and DAF-7 TGF $\beta$  signaling pathways (Bretscher et al. 2008; Hallem and Sternberg 2008). These signaling pathways are involved in nutritional signaling during dauer formation (see Fielenbach and Antebi 2008 for review). The nuclear hormone receptor NHR-49, which is involved in transcriptional regulation of fat metabolism (Van Gilst et al. 2005), is also required for CO<sub>2</sub> avoidance (Hallem and Sternberg 2008), although it is unclear whether starvation changes NHR-49 expression. Finally, C. elegans modulates CO<sub>2</sub> avoidance by integrating sensory information on food and  $O_2$  levels. During hypoxia, low levels of  $O_2$  prevent activation of the  $O_2$ -dependent prolyl hydroxylase, EGL-9, thereby preventing the proteolytic degradation of the transcription factor HIF-1 (Epstein et al. 2001; Jiang et al. 2001). egl-9 mutants do not avoid CO<sub>2</sub> (in fact, they are attracted to  $CO_2$ ), but this defect is suppressed by loss of function in *hif-1*. Furthermore, wild-type animals that have been exposed to hypoxic conditions also stop avoiding CO<sub>2</sub>. When C. elegans are presented with a choice between being attracted to  $O_2$  or repelled by  $CO_2$ , their decision is modulated by the presence or absence of food, as well as their genotype. In strains with an NPR-1 neuropeptide receptor with the 215V polymorphism (which is found in solitary feeding strains such as the laboratory wild-type reference strain N2), CO<sub>2</sub> avoidance prevails over O<sub>2</sub> attraction. In contrast, in strains with the 215F polymorphism (such as the social feeding CB4856 Hawaiian isolate), the presence of food causes O<sub>2</sub> response to dominate and the absence of food causes the CO<sub>2</sub> response to dominate (Bretscher et al. 2008).

#### **1.3.2 OTHER MODULATORY NEUROTRANSMITTERS**

The catecholamines dopamine, tyramine, and octopamine also play roles in modulation of response to octanol, although the details are less clear. Dopamine seems to dampen signaling in the ASH neuron response to octanol. *rgs-3* mutants are defective for response to high concentrations of octanol, due to excessive signaling in ASH neurons (see above; Ferkey et al. 2007). This behavioral defect can be suppressed by decreasing the function of *cat-2*, which encodes the *C. elegans* homolog of tyrosine hydroxylase and is essential for dopamine biosynthesis (Lints and Emmons 1999). Other investigators have found that while serotonin potentiates response to octanol, exogenous dopamine suppresses this serotonin-dependent potentiation, consistent with a role for dopamine in dampening signaling (Wragg et al. 2007). Tyramine and octopamine negatively modulate octanol response as well. Octopamine probably acts directly on ASH neurons via the octopamine receptor coded by designated gene

F14D12.6, whereas tyramine probably acts indirectly on the octanol response circuit via the receptor TYRA-3, which is expressed in the dopaminergic ADE/CEP neurons (Wragg et al. 2007).

#### **1.3.3** TEMPERATURE

*C. elegans* is able to detect and respond to changes in temperature (reviewed in Mori et al. 2007), and behavioral response toward soluble attractants is modulated by temperature, but the mechanism of this modulation is unclear. Temperature is sensed by the AFD neurons (Mori 1999). In response to step changes in temperature, Ca<sup>2+</sup> influx increases transiently in AFD neurons (Kimura et al. 2004; Ramot et al. 2008). *C. elegans* are attracted to NaCl sources most effectively at temperatures  $\pm 5^{\circ}$ C of the temperature at which they are raised (Dusenbery et al. 1978). Na<sup>+</sup> and Cl<sup>-</sup> ions are sensed by the functionally asymmetric ASEL and ASER sensory neurons, respectively. One study has suggested that ASEL and ASER are modulated by temperature differently (Adachi et al. 2008). Interestingly, AWC-mediated attraction to isoamyl alcohol does not seem to be affected by temperature (Adachi et al. 2008).

#### **1.4 HOW DO CAENORHABDITIS ELEGANS NAVIGATE?**

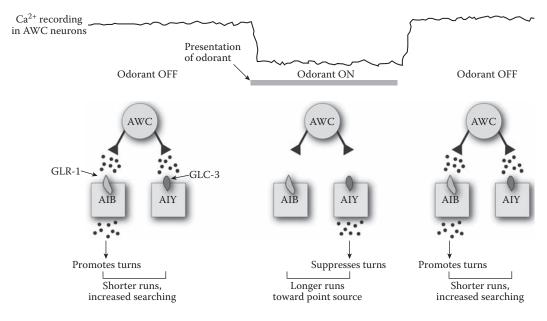
*C. elegans* transitions between three general states of locomotion (Gray et al. 2005; Wakabayashi 2004; Zhao et al. 2003). These states are best understood in the context of foraging behavior (i.e., food searching). In the presence of food, *C. elegans* moves forward slowly, and turns or reversals are very frequent. These reversals are generally short in duration, which results in lower turn angles. This behavior is termed dwelling, and allows animals to stay in the vicinity of food. Initially, when animals are removed from food, the frequency of reversals remains high, but turn angles increase, resulting in larger direction changes. Furthermore, their rate of locomotion increases tenfold. This results in a rapid scan of the local environment for food in a behavior termed area-restricted search (Hills et al. 2004). When animals are deprived of food for longer periods of time, reversal rates decrease whereas speed remains high; this results in dispersal, which presumably allows animals to move away from an exhausted food source.

How is chemotaxis impacted by these changes in feeding and locomotion status? Taxis toward a point source of any attractant can be essentially thought of as an exercise in how gradients are interpreted by a sensory system: in other words, how an organism understands its spatial orientation relative to its environment. Detailed motion-tracking and behavioral analysis suggests that *C. elegans* navigates gradients using a strategy called a biased random walk for salt chemotaxis (Pierce-Shimomura et al. 1999; Ryu and Samuel 2002; Zariwala et al. 2003). As animals increase forward movement and decrease turns, they tend to move away from a previous location, and as they decrease forward movement and increase turns, they tend to stay in the same general location. Changes in locomotory patterns are controlled by sensory input via chemosensory neurons (Gray et al. 2005; Wakabayashi 2004).

The activity of the AWC-mediated chemotaxis toward volatile attractants has been characterized at the level of the neural circuit in detail (Chalasani et al. 2007). Using the analogy of retinal bipolar cells again (Yang 2004) (see Figure 1.6), AWC neurons function as OFF-sensing neurons; that is, AWC neurons are tonically active in the absence of odorant, are suppressed in the presence of odorant, and are stimulated by odorant removal. AWC neurons form glutamatergic synapses onto the AIB and AIY neurons. AIB neurons are also OFF-sensing neurons; they receive excitatory glutamatergic input via the AMPA/kainate-like receptor GLR-1. Activation of AIB neurons by odorant removal results in more turning, whereas inactivation of AIB by the presence of odorant results in less turning. In contrast, the AIY neurons are ON-sensing neurons. They receive inhibitory glutamatergic input via the chloride-gated glutamate channel GLC-3 (Horoszok et al. 2001). Inhibition of AIY neurons by odorant removal results in more turning, whereas activation of AIY by the presence of odorant results in less turning. The net result is longer runs (fewer turns) toward a point source of odorant when animals are moving up a concentration gradient of odorant and shorter runs (more turns) when animals are moving down a concentration gradient (Figure 1.7).

It is interesting to note the many parallels between AWC-AIB-AIY synapses and photoreceptorbipolar OFF-bipolar ON synapses. Sensory neurons in both systems use GPCR signaling involving  $G_i\alpha$ -like proteins, receptor-like transmembrane guanylyl cyclases, and cGMP-gated channels. Both systems have neurons that do not spike with action potentials, but use tonic-graded neurotransmitter release, which is well suited for detecting graded stimuli such as a gradient of odorant emanating from a point source. Additionally, both systems use glutamate as both an excitatory and inhibitory neurotransmitter (although mammals utilize inhibitory metabotropic glutamate receptors and nematodes use hyperpolarizing chloride-gated channels). It will be interesting to determine how other sensory modalities integrate into chemotaxis. For instance, salt-sensing and temperature-sensing neurons also synapse onto AIB and AIY interneurons (White et al. 1986; Chen et al. 2006), and these two types of stimuli can modulate chemotaxis.

The asymmetrical salt-sensing ASEL and ASER neurons are also organized in an ON–OFF manner (Suzuki et al. 2008). ASEL neurons, which function as ON-sensing neurons, sense Na<sup>+</sup> and exhibit a transient Ca<sup>2+</sup> increase when an increase in Na<sup>+</sup> levels is detected. ASER neurons, on the other hand, function as OFF-sensing neurons; they sense Cl<sup>-</sup> and exhibit a transient Ca<sup>2+</sup> increase when a decrease in Cl<sup>-</sup> levels is detected. Activation of ASEL increases forward movement, whereas activation of ASER increases turning. Thus, navigation toward salt uses the same general principle as navigation toward point sources of volatile odorants. It is unclear whether the difference between tonic activation of AWC neurons and transient activation of ASE neurons is physiologically relevant, as different handling techniques were used to immobilize *C. elegans* for imaging in these two studies (Chalasani et al. 2007; Suzuki et al. 2008).



**FIGURE 1.7** *C. elegans* navigation up a concentration gradient toward a point source of attractant. AWC and AIB neurons are OFF-sensing neurons and are active in the absence of odorant; AIY neurons are ON-sensing neurons and are active in the presence of odorant. As glutamate is released from AWC, AIB is depolarized via the AMPA/kainate-like glutamate receptor, GLR-1, and AIY is hyperpolarized via the glutamate-gated Cl<sup>-</sup> channel, GLC-3. AIB activity (when odorant levels decrease) promotes turning, which increases searching; whereas AIY activity (when odorant levels increase) suppresses turning, which increases runs toward a point source. (Adapted from Chalasani, S.H. et al. *Nature* 450, 63–70, 2007 and Gray, J.M., J.J. Hill, and C. Bargmann. *Proc. Natl. Acad. Sci. USA* 102, 3184–91, 2005.)

#### 1.5 WHAT DO CAENORHABDITIS ELEGANS SMELL?

*C. elegans* has traditionally been used as a model organism in the laboratory. Hence, little is known about its natural history and ecology (Kiontke 2006). As *C. elegans* becomes more familiar to researchers outside the original *C. elegans* community, some interesting studies have emerged on how *C. elegans* olfaction might play a role in the greater world outside a Petri dish and an incubator.

#### **1.5.1 OLFACTORY RESPONSES IN FORAGING**

An innate behavior of *Caenorhabditis* nematodes is to search for food. Although little is known about what it eats in the wild, it seems reasonable to assume that a major source of food is various forms of soil bacteria. The olfactory system of *C. elegans* obviously plays a role in finding food and there is innate preference for certain types of bacterial odors. For instance, *C. elegans* is strongly attracted to the pathogenic soil bacteria *Serratia marcesens* (Zhang et al. 2005), despite the fact that *S. marcesens* is actually toxic to *C. elegans*. This incongruity suggests that the interaction of *C. elegans* and bacteria may be as complex as other prey/predator interactions.

The chemical cues produced by bacteria that are innately attractive to *C. elegans* are not well studied. One early study suggested that ammonium ions, which are attractive to *C. elegans* as both soluble and volatile odorants (Frøkjaer-Jensen et al. 2008), are produced by some attractive bacterial species, such as *E. coli* and the pseudomonad species *Pseudomonas aeruginosa* and *P. fluorescens* (Andrew and Nicholas 1976). Another candidate class of chemical attractants is acylated homoserine lactones (AHSLs), signaling molecules secreted by Gram-negative bacteria that utilize quorum-sensing (QS) systems. QS is a signaling mechanism employed by bacteria wherein molecules called autoinducers (such as AHSLs) are expressed at low levels by bacterial cells. As cell density increases, autoinducer concentration increases, which leads to a positive feedback loop and increased autoinducer expression. The autoinducer then activates signaling pathways, leading to physiological changes in bacteria (reviewed in Von Bodman et al. 2008). Purified AHSLs are weak chemoattractants for *C. elegans* (Beale et al. 2006). This makes sense, as the presence of AHSLs would indicate a potentially abundant food source. Chemotaxis toward other identified QS molecules, such as quinolones and boronated furanones, has not been tested.

There are also molecules made by bacteria that are inherently repulsive to C. elegans. Certain strains of S. marcescens produce cyclic lipopentapeptides called serrawettins. Serrawettins are biosurfactants that are essential for a type of bacterial behavior called swarming motility (Matsuyama et al. 1992). When lawns of E. coli bacteria mixed with the serrawettins W2 or W3 are spotted onto Petri dishes, naive C. elegans animals avoid E. coli. A mutant S. marcescens strain that does not produce serrawettin W2 is not repulsive to C. elegans, even though the bacteria remain pathogenic for C. elegans. The related serrawettin W1 does not alter the animal's preference for E. coli. This suggests that avoidance of specific serrawettins is a specific chemosensory response and not a response to general changes in surface tension caused by a biosurfactant or general pathogenicity of the bacteria. This avoidance response is mediated by the AWB neurons (which are known to detect volatile repellants) via cGMP signaling through the TAX-2/TAX-4 cGMP-gated channels. The context of chemical presentation is also important. In contrast to the Petri dish-based population assay described above, serrawettins W1, W2, and W3 induce acute avoidance responses when they are directly applied to the animal in a liquid drop. Surfactin, a biosurfactant produced by Bacillus subtilis, repels C. elegans in the plate assay, but is inactive in the drop assay (Pradel et al. 2007). Collectively, these results suggest that while some responses to bacterial metabolites are innate, others are highly context-dependent and may involve factors such as the immediate chemical environment and feeding state.

*C. elegans* also learns to avoid toxic bacteria after pre-exposure, a process that requires serotonin (Zhang et al. 2005). There is some plasticity in this "learned behavior." As mentioned above, acylated homoserine lactone (ASHL) autoinducer compounds are weak attractants for naive *C. elegans*,

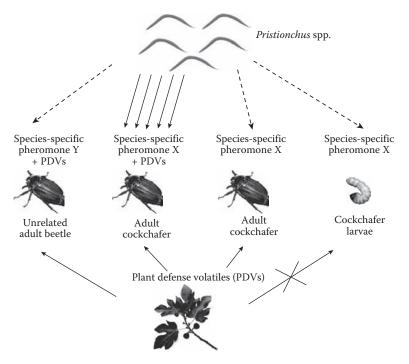
but when they are paired with the pathogenic *Pseudomonas* strain, PAO1, AHSLs become weakly repulsive (Beale et al. 2006). Thus, other sensory stimuli (e.g., gustatory stimuli and/or innate immune responses) can influence the response of *C. elegans* to bacterial-specific odorants.

#### **1.5.2 OLFACTORY INTERACTIONS WITH OTHER ORGANISMS**

Nematodes represent an extremely diverse phylum, with individual species occupying distinct ecological niches (Kiontke and Sudhaus 2006). Thus, there are probably species-specific evolutionary adaptations to olfactory behavior that facilitate particular lifestyles. One remarkable example of this is in the olfactory behavior of genus Pristionchus nematodes, of which one species, P. pacificus, has now been sequenced (Dieterich et al. 2008). Pristionchus was previously grouped into Diplogastridae (Blaxter et al. 1998), but more recently in Rhabditidae (Sommer 2006; Kiontke and Fitch 2005). Pristionchus nematodes have recently been found to adapt a necromenic lifestyle. Unlike true parasites that feed off live hosts, necromenic *Pristionchus* nematodes feed off the corpses of their host insects (Pristionchus also feeds on bacteria, similar to C. elegans). P. maupasi is one species from this genus that feeds off the corpses of adult cockchafer beetles (Melolontha spp.) (Hong et al. 2008a). Cockchafers spend three years in pupal and larval stages, and then metamorphose into a short-lived, three-week adult stage, which is the preferred host for P. maupasi. In chemotaxis assays, P. maupasi is strongly attracted to cuticle washes from adult, but not larval cockchafers. There are at least two classes of chemical components from the cuticle washes that are chemoattractants. First is the cockchafer sex pheromone phenol, which attracts P. maupasi but not related Pristionchus species. Second are plant defense volatiles, including compounds such as green leaf alcohol ((Z)-3-hexen-1-ol, or GLA) and linalool. These are chemicals whose production is upregulated by plants when they are grazed upon by herbivorous insects, and their presence strongly synergizes the chemoattraction to phenol in the nematodes. Only the adult cockchafers feed upon these plants and they release a small amount of plant defense volatiles that they ingest. Since only the adult cockchafers feed on plants, and since *P. maupasi* prefer adult cockchafers, the sex pheromone phenol acts as a species-specific cue, whereas the plant defense volatiles act as a temporal cue to indicate adulthood of the host insect (Figure 1.8).

Parasite-host specific interactions between different *Pristionchus* species and insects probably involve other species-specific odorants, as different species show different chemoattractive profiles for odorants typically associated with their natural habitats and insect hosts (Hong and Sommer 2006). In *P. pacificus*, the homolog of *C. elegans* cGMP-activated protein kinase (PKG) EGL-4 may be required for attraction to the pheromone ETDA (Hong et al. 2008b). Natural variation in the *P. pacificus* strains isolated from different parts of the world (Hong et al. 2008b), similar to how natural variation in NPR-1 accounts for variation in feeding behavior in *C. elegans* (de Bono and Bargmann 1998) (Figure 1.8).

From the reverse perspective, other organisms may exploit the chemosensory behavior of nematodes. For instance, the roots of legume plants such as *Cajanus cajan* release soluble flavonoids that attract nitrogen-fixing symbiotic rhizobacteria to their root nodules (Pandya et al. 1999). However, since soluble cues can only act at a short distance (a few millimeters), they are probably insufficient to attract rhizobacteria to root nodules from greater distances. *C. elegans* are attracted to the legume *Medicago truncatula*, whereas they are indifferent to *Arabidopsis*, a nonlegume plant. This attraction is mediated by the volatile attractant, dimethyl sulfide, which is released by *M. truncatula* (but not *Arabidopsis*) and can presumably act at longer distances. *C. elegans* that have been grazing on *S. melioti* can populate the root nodules of aseptically grown legumes with the rhizobacteria under laboratory conditions (Horiuchi et al. 2005). While it is unknown whether *C. elegans* and *M. truncatula* occupy similar ecological niches in nature, it is known that agriculturally cultivated legumes initially lacking symbiotic rhizobacteria eventually acquire rhizobacteria over time (Purchase and Nutman 1957). These experiments suggest that soil nematodes might play a role in enriching and dispersing microbial diversity within the rhizosphere.



**FIGURE 1.8** Host species and lifestage specificity in *Pristionchus* nematodes. The nematodes are attracted to two different attractants: beetle species-specific pheromones and plant defense volatiles (PDVs). This second attractant serves as a temporal cue for the nematodes, because only adult cockchafer beetles leave the soil to forage for plant material. Each attractant itself is weak, but the two combined synergize to form a strong attractive signal. (From Beetle larvae image © Horla Varlar; used under Creative Commons Attribution 3.0 License. Nematode image © Zeynep Altun, used under Creative Commons Attribution ShareAlike 2.5 License. Adult cockchafer beetle image © Jon Law, used with permission.)

### 1.6 CONCLUDING REMARKS

Understanding a nervous system, even the relatively small *C. elegans* nervous system, is no small task. Clearly, the analysis of the nervous system of *C. elegans* is allowing researchers to identify the basic mechanisms involved in chemosensation and related behaviors. Forward genetic approaches like those used in *C. elegans* excel at identifying previously unsuspected mechanisms and pathways. We can expect the invertebrate communities to continue to lead the field of neuroscience in defining the basic mechanisms of cellular and molecular behavioral neuroscience. However, completely understanding the cellular and molecular basis of behavior will require the neuroscience community to integrate information obtained from disparate sources and experimental approaches. This becomes more difficult as we learn more, in part because of the large amount of information even the *C. elegans* community has generated. A major challenge for the next decade will not just be continuing to define the basic principles of neuronal function, but also consolidating the data into useful models and databases.

### ACKNOWLEDGMENTS

We thank Gareth Harris and Rick Komuniecki for sharing unpublished data, Paul Orwin, John Skillman, and Dave Polcyn for useful discussions, Morris Maduro and Dan Bumbarger for critical reading of the manuscript, and Zeynep Altun, Jon Law, and Horla Varlan for sharing images.

This work was supported in part by the National Institute of Drug Abuse (M.Y.C.) and the National Institute of General Medical Sciences (A.C.H.). All images © Michael Chao except where noted; images are released under the Creative Commons Attribution-Share Alike 3.0 License (see http://creativecommons.org/licenses/by-sa/3.0/us/ for details). Color versions of images are available upon request.

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# 2 Odor Coding in Insects

C. Giovanni Galizia and Silke Sachse

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# 2.1 INSECT OLFACTORY RECEPTORS

Most organisms rely on their olfactory system to detect and analyze olfactory cues in the environment, cues that are subsequently utilized in the context of behavior. Odorants are recognized by olfactory sensory neurons (OSNs), which are located in the olfactory epithelia of vertebrates or in the dendrites of olfactory sensory cells within the sensilla on the antennal surface of insects (Buck and Axel 1991; Carlson 2001; Chess et al. 1992; Vosshall et al. 2000). The OSNs express odorant receptors (ORs), which are related to seven transmembrane G-protein-coupled receptors (GPCR)

and transduce odorant binding to cellular excitation (see also Chapter 7). The olfactory system of the genetic model organism, the fruit fly Drosophila melanogaster, has been the focus of numerous investigations. Drosophila has two pairs of olfactory organs, the antennae and the maxillary palps. Each antenna contains about 1200 OSNs housed in a total of 410 olfactory sensilla covering the antenna, while the maxillary palp has about 120 OSNs and 60 olfactory sensilla (Laissue and Vosshall 2007). The sensilla are of three morphological types: basiconic sensilla, trichoid sensilla, and coeloconic sensilla (Venkatesh and Singh 1984). Across insects, there is a beautiful diversity of sensillar morphologies, including pore plate sensilla, campaniform sensilla, sensilla ampullacea, and more (Kleineidam and Tautz 1996; Steinbrecht 1996). In recent studies, the OR gene family of D. melanogaster has been identified and shown to comprise 62 defined members (Table 2.1) (Clyne

#### List of Sensilla, OR type, Innervated Glomerulus, and Best Ligand for Drosophila Melanogaster Sensillum Glomerulus **CR** Type **Best Ligand** Trichodea (Antenna) at1 Or67d DA1, VA6 cis-Vaccenyl acetate at2 Or23a DA3 1-Pentanol at2 Or83c DC3 at3 Or2a DA4m Isopentyl acetate at3 Or19a, Or19b DC1 2-Octanone, 1-octen-3-ol at3 Or43a DA41 1-Hexanol Or47b VA11m at4 Or65a, Or65b, Or65c DL3 Pyrrolidine at4 VA1d at4 Or88a **Basiconica** (Antenna) ab1A Or42b DM1 Ethyl propionate, ethyl acetate ab1B Or92a VA2 2,3-Butanedione ab1C Gr21a V Carbon dioxide ab1D Methylsalicylate Or10a, Gr10a DL1 ab2A Or59b DM4 Methyl acetate Or33b, Or85a ab2B DM5 Ethyl 3-hydroxybutyrate ab3A Or22a, Or22b DM2 Ethyl hexanoate Or85b ab3B VM5d 6-Methyl-5-hepten-2-one ab4A Or7a DL5 E2-Hexenal ab4B Or33a, Or56a DA2 ab5A Or82a VA6 Geranyl acetate ab5B DM3 Or33b, Or47a Pentyl acetate ab6A Or13a DC2 1-Octen-3-ol ab6B Or49b VA5 2-Methylphenol ab6B Or98b VM5d ab7A Or98a VM5v Ethyl benzoate ab7 Or67c VC4 (VC3m) Ethyl lactate ab8A Or43b VM2 Ethyl trans-2-butenoate ab8B Or9a VM3 3-Hydroxy-2-butanone ab9 Or67b VA3 Acetophenone ab9 Or69aA, Or69aB D

DL4

DM6

2-Heptanone, acetophenone

Phenylethyl alcohol

# **TABLE 2.1**

ab10

ab10

Or49a, Or85f

Or67a

IABLE 2.1	(Continued)		
Sensillum	СК Туре	Glomerulus	Best Ligand
		Coeloconica (Antenna)	
ac1	Ir31a		Ammonia
ac1	Ir75d		Ammonia
ac1	Ir92a, Ir76b		Ammonia
ac2	Ir75a		1,4-Diaminobutane
ac2	Ir75d		1,4-Diaminobutane
ac2	Ir76b		1,4-Diaminobutane
ac3	Ir75a, Ir75d		Propanal
ac3	Or35a, Ir76b	VC31	Hexanol
ac4	Ir84a		Phenylacetaldehyde
ac4	Ir75d		Phenylacetaldehyde
ac4	Ir76a, Ir76b		Phenylacetaldehyde
		Basiconica (Palp)	
pb1A	Or42a	VM7	Propyl acetate
pb1B	Or71a	VC2	4-Methylphenol
pb2A	Or33c, Or85e	VC1	(-) Fenchone, cyclohexanone
pb2B	Or46aA	VA71	4-Methylphenol
pb3A	Or59c	1	
pb3B	Or85d	VA4	2-Heptanone

#### TABLE 2.1 (Continued)

*Note:* The best ligands are to be taken with caution: in many cases, this is the substance that gives the best response of all tested substances, but the response is small, and it is likely that another substance will elicit much stronger responses. Mapping of coeloconic sensilla neurons to AL glomeruli has not yet been published.

et al. 1999; Gao and Chess 1999; Vosshall et al. 1999). Several studies have been dedicated to characterize the molecular receptive ranges of identified ORs (Dobritsa et al. 2003; Hallem and Carlson 2006; Pelz et al. 2006).

ORs are expressed following a conserved pattern in *Drosophila* as well as in mammals (see Chapter 7). Every OSN typically expresses only one type of OR (as well as the ubiquitous Or83b, see below). However, a given OSN can also coexpress up to three conventional ORs determining a specific molecular response profile along with the Or83 coreceptor (Table 2.1). OSNs expressing the same type of OR, converge to a single glomerulus in the antennal lobe (AL), which represents the first olfactory neuropil in the insect brain (see below). However, a few cases of 1:2 and 2:1 innervation ratios in *Drosophila* have also been described (Couto et al. 2005; Fishilevich and Vosshall 2005).

Interestingly, ORs in *Drosophila* possess no significant homology to other known GPCRs. However, the total length of the receptor proteins and the size of the internal and external loops are similar to most members of the GPCR protein family (Clyne et al. 1999). The identified genes of the OR family are highly divergent, even among drosophilid species. At least two receptors generate alternative splicing products (Clyne et al. 1999; Robertson et al. 2003).

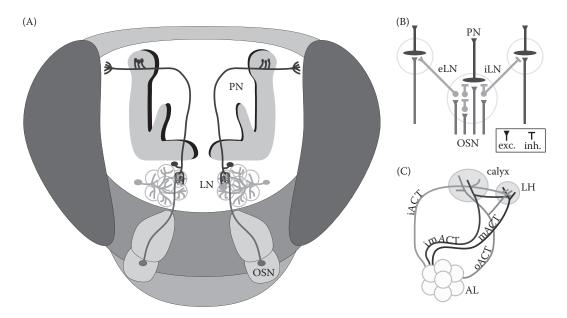
While the functional organization of the olfactory system in vertebrates and insects shows clear similarities, the atypical heteromeric and topological design of the ORs in *Drosophila* appears to be insect-specific. Almost all OSNs express a chaperon receptor, called Or83b. Or83b is highly conserved in many insect species (Dahanukar et al. 2005), and it is also possible to functionally exchange Or83b with orthologous Or83b proteins from other insects (Hill et al. 2002; Jones et al. 2005; Krieger et al. 2003; Pitts et al. 2004).

Or83b has a membrane topology that differs from the related GPCR topology, with the N-terminus and the most conserved loops located in the cytoplasm (Benton et al. 2006). Furthermore, Or83b has a 70-amino acid long insert in the second intracellular loop, which might be responsible for the distinct function of Or83b vis-à-vis the remaining members of the insect OR family (Benton et al. 2006). In recent studies, it was shown that Or83b is a nonselective cation channel that interacts directly with the endogenous ligand-tuned OR (Sato et al. 2008; Wicher et al. 2008). In addition, Or83b is needed for the functional integration of receptor proteins in the dendritic part of the OSNs within the sensillum shaft (Larsson et al. 2004; Neuhaus et al. 2005).

The possible role of the G-proteins expressed in OSNs remains unclear, and the involvement of G-proteins in the signal pathway is a controversial topic. While Sato et al. (2008) give evidence primarily for a direct ligand gating of the receptor complex, Wicher et al. (2008) show that the Or83b channel is also activated by internal cAMP or cGMP. The activation of the G-protein-coupled signal cascade appears considerably slower than the ionotropic response of the receptor complex. The G-protein-coupled signal cascade modulates the activation of the odorant through either cAMP and the subsequent opening of cyclic nucleotide-gated channels or through an IP<sub>3</sub>-mediated signal cascade (Krieger and Breer 1999). In OSNs of the maxillary palps, a phospholipase C could be substantiated as an important part of the IP<sub>3</sub>-signal pathway (Riesgo-Escovar et al. 1995). Even though molecular response profiles are known for many *Drosophila* receptors, the interaction of ORs with the component of the signal cascade is not yet well understood. An important task will be to localize both the binding sites of ORs with the odor ligand, and with the intracellular signaling components. Furthermore, it has to be solved which domains of the ORs and the Or83b channel interact with each other, and how this interaction leads to a functional cellular response.

Not all OSNs express ORs that are related to GPCRs. In *Drosophila*, a recent study has identified the receptors that are expressed in coeloconic sensilla. These receptors are related to the gene family encoding ionotropic glutamate receptors and were therefore named ionotropic receptors (IRs) (Benton et al. 2009). Their sequence shows that they do not belong to the hitherto described kainate, AMPA, or NMDA classes. IRs are expressed in a combinatorial fashion in OSNs housed in coeloconic sensilla that respond to many distinct odors, but do not express either insect ORs or gustatory receptors.

Another important player in the primary odor response consists of a family of odorant binding proteins (OBPs). OBPs are assumed to guide the odorous molecules through the sensillum lymph to allow interaction with a specific OR in the dendritic membrane of the OSNs, but more complex models of their action draw a multifunctional picture (Kaissling 2001). Evidence from various insect species indicates a complex interplay of several sensory molecules in the subsequent pheromone reception and transduction process (Vogt 2003; Vosshall 2008). While specialized pheromone binding proteins (PBPs) are supposed to shuttle hydrophobic pheromone molecules through the sensillum lymph toward specific receptors (Leal 2003; Xu et al. 2005), a so-called sensory neuron membrane protein is suggested to stabilize a functional receptor complex or dock PBPs to the receptor site (Vogt 2003; Jin et al. 2008). The former concept that a PBP just passively transports pheromones to sensory cells has been challenged by studies showing that PBPs specifically interact with pheromones and undergo distinct conformational changes (Mohl et al. 2002; Grosse-Wilde et al. 2006; Laughlin et al. 2008). Interestingly, the conformational change of a PBP has been found to be sufficient for neuronal activation, suggesting a direct interaction of a pheromone/PBP complex with the receptor (Laughlin et al. 2008). One identified member of the PBP family is the OBP, LUSH, which is expressed and secreted exclusively by non neuronal support cells in trichoid sensilla (Kim et al. 1998; Shanbhag et al. 2005). A study by Xu et al. (2005) shows that LUSH is required for activation of pheromone-sensitive OSNs in Drosophila. LUSH mutants lack the detection of the pheromone, cis-vaccenyl acetate, at the physiological as well as at the behavioral level. Unlike the situation in vertebrates, where OBPs are present in the olfactory mucosa and thus potentially in contact with all OSNs, the compartmentalization of OSNs into sensilla gives insects a much more specific control: OPBs are selectively present in particular sensilla, and are more likely to contribute to odor-specific response profiles.



**FIGURE 2.1** Olfactory system in the fruit fly, *Drosophila melanogaster*. (A) Schematic view of the fly head, with a cut-open brain. Olfactory sensory neurons (OSNs) are located on the antennae and project into the antennal lobe, where they interact with local neurons (LN), and synapse onto projection neurons (PN). These target the mushroom bodies and the lateral protocerebrum. (B) Synaptic connectivity scheme, showing OSN input to a glomerulus, where OSNs make direct contact onto PNs, or indirect contact via LNs, which can be inhibitory and excitatory, and connect either within a glomerulus or across glomeruli. (C) Schematic view of the half brain (midline is left) with the four PN tracts (iACT, imACT, mACT, oACT) in *Drosophila* that connect the antennal lobe (AL) to the mushroom body calyx and the lateral horn (LH).

#### 2.2 THE ANTENNAL LOBE (AL)

The first neuropil in the insect brain that processes olfactory information is the AL, a structure common to all insects, and secondarily lost in some anosmic species (Strausfeld and Hildebrand 1999). It is analogous in structure and function to the vertebrate olfactory bulb, but evolved independently (Strausfeld and Hildebrand 1999). However, unlike in mammals, insect neurons generally have their cell bodies outside the brain, and synaptic computation is accomplished entirely in the somaless neuropil. Similarly, while the mammalian bulb is structured in layers (glomerular layer, soma layer, etc.,) the insect AL is structured entirely in glomerular units, which are the interaction sites of OSNs, local neurons (LNs), projection neurons (PNs), and others (Figure 2.1A and B). Most synaptic contacts are within olfactory glomeruli (Boeckh and Tolbert 1993; Gascuel and Masson 1991).

Even though the structure and function of ALs appears close to universal, there is considerable diversity of AL organization across insects (Schachtner et al. 2005). Indeed, insects have an evolutionary history of over 400 million years, and most modern insect orders were already present 250 million years ago, allowing for considerable divergent evolution (Grimaldi and Engel 2004). Thus, glomerular arrangements, numbers, position of soma groups, neuron populations and more, differ across species. Several examples will be considered in the following section.

### 2.2.1 SENSORY NEURON AXONS

OSN numbers differ among species. *Drosophila* has ~1200 OSNs in both sexes (Stocker et al. 1990). *Manduca* have ~300,000 OSNs on each antenna (Oland and Tolbert 1988; Sanes and Hildebrand 1976b). Male honeybees (drones) have ~300,000 OSNs, while (female) worker bees have ~65,000 (Esslen and Kaissling 1976). Hemimetabolous insects increase the number of receptors with each nymphal instar (Chapman 2002; Ochieng et al. 1998; Schafer and Sanchez 1973). Adult cockroaches have ~150,000 OSNs (Ernst et al. 1977), locusts have between 50,000 (Ernst et al. 1977) and 105,000 (Anton et al. 2002) OSNs as adults.

OSN axons project to the AL via the antennal tract(s). In honeybees, there are four tracts, T1–T4 (Suzuki 1975). T1 innervates ~70 glomeruli, T2 ~7, T3 ~70, and T4 ~7 glomeruli (Arnold et al. 1983; Flanagan and Mercer 1989a; Galizia et al. 1999a). This segregation corresponds to distinct groups of PNs that leave the AL following different tracts (see below) (Abel et al. 2001; Kirschner et al. 2006). At this time, the functional significance of this segregation remains unknown. Mechanosensory and gustatory axons from the antenna also use the antennal tracts and bypass the AL toward the antennal mechanosensory and motor center in the dorsal lobe (Gewecke 1979; Kloppenburg et al. 1997; Mobbs 1982; Suzuki 1975). Efferent motor neurons from the dorsal lobe innervate the antennal muscles and use the antennal nerves to enter the antenna, together with axons from modulatory neurons, which most likely use biogenic amines as transmitters. The function of these modulatory neurons has not yet been elucidated directly, but an effect of biogenic amines on OSN responses has been shown (Pophof 2002).

#### 2.2.2 PROJECTION OF OLFACTORY SENSORY NEURONS (OSNs) INTO THE ANTENNAL LOBE (AL)

Each OSN axon innervates a single ipsilateral glomerulus in the AL in most species. In flies, OSNs form an axonal commissure between the two Als, and individual axons innervate both homologous glomeruli (Stocker 1994; Strausfeld 1976). Each bilaterally innervated glomerulus receives equal input from both antennae (Vosshall et al. 2000). A few glomeruli in *Drosophila* are innervated unilaterally. These are the glomeruli V, VL1, VP1, as well as VP2 and VP3, which collect input from sensilla located on the *Drosophila* arista (Stocker 2001; Stocker et al. 1983). OSNs may innervate the entire glomerulus (e.g., those innervating T4 glomeruli in bees), or just the glomerular periphery (as the other bee glomeruli, or many fruit fly glomeruli). The innervation may follow the antennal topology: in bees, OSNs from the distal antennal segments innervate the outer layer of the glomerular cap, and more proximal OSNs innervate the central layers (Pareto 1972). Somatotopic projections are also known from *Manduca* (Christensen et al. 1995) and *Periplaneta* (Hösl 1990).

#### 2.2.3 TARGETING MECHANISMS OF OLFACTORY SENSORY NEURONS (OSNs)

How do receptor axons find the right glomerulus? The molecular mechanisms have been studied in *Drosophila*, where the relationship OR to innervated glomerulus is best known (Couto et al. 2005; Fishilevich and Vosshall 2005). Generally, each OSN expresses one (or sometimes a few) OR, and OSNs that express the same OR converge on a single glomerulus in each hemisphere (Vosshall et al. 2000). Examples of OSNs that express more than one OR include dOr33c and dOr85e, which are coexpressed in pb2a OSNs (*palp basiconic type 2*, neuron *a*), and where both contribute to these neurons' odor responses (Goldman et al. 2005). dOr22a and dOr22b are coexpressed in ab3a neurons (*antennal basiconic sensillum 3*, neuron *a*), but no functional role for dOr22b has yet been found (Dobritsa et al. 2003). OSNs expressing dOR67d innervate the two glomeruli DA1 and VA6 (Fishilevich and Vosshall 2005).

While ORs are important for axon targeting to the glomerulus in mammalian OSNs (Feinstein et al. 2004; Feinstein and Mombaerts 2004; Wang et al. 1998), this is not the case in insects (Dobritsa et al. 2003). Several transcription factors are known to be required for correct OSN targeting in *Drosophila* (Rodrigues and Hummel 2008). These include the Src homology domain 2 (SH2)/SH3 adapter Dock (Ang et al. 2003), the serine/threonine kinase Pak (Ang et al. 2003), the cell surface proteins N-cadherin (Hummel and Zipursky 2004), the POU domain transcriptional factor Acj6 (Komiyama et al. 2004), and the immunoglobulin Dscam to be expressed in PNs or LNs (Hummel

et al. 2003; Zhu et al. 2006). The transmembrane protein, semaphorin-1a, does not mediate largescale target finding, but it does mediate short-range precision and axon convergence into a single glomerulus (Lattemann et al. 2007). Probably, some sort of combinatorial mechanism of these factors is used for identity/target determination. For example, dOr22a targeting is independent of Dscam, but in other axons, Dscam mutation disrupts OSN targeting either partially or completely (Hummel et al. 2003). Acj6 is expressed in all OSNs in the antenna, but only in some in the maxillary palps. Axon-axon interaction is necessary for glomerular convergence (Komiyama et al. 2004), and glial cells are necessary too (see below) (Tolbert et al. 2004). The identity of the glomerulus itself, however, is not determined by OSNs, but rather by PNs that form "protoglomeruli" (Jefferis et al. 2004).

#### 2.2.4 ANTENNAL LOBE (AL) GLOMERULI

Almost all animals, whether insects or mammals, have evolved olfactory glomeruli (Hildebrand and Shepherd 1997). Even though, on a small scale, the neighborhood relationship of glomeruli may follow a logical rule dictated by their molecular response profile (Sachse et al. 1999), this is not a general rule (Linster et al. 2005). Thus, glomeruli might reflect that there is no physical property common to all odors that can be mapped onto the two dimensions of the brain surfact. This differs from spatial position of visual stimuli, or frequency coding for sounds, cases in which topological arrangements are known (brain maps). However, not all sensory patterns that have physically defined dimensions are reflected with a corresponding topology in the brain. Colors, for example, derive from the spectral properties of photon wavelength, but rather than following this linear arrangement, all color-vision systems in animals create artificial dimensions using different colorsensitive photoreceptors and creating subsequent color-opponency channels (e.g., the green-red and the yellow-blue dimensions in humans). The glomerular organization in olfaction may reflect the multidimensionality of olfaction *per se*, or just reflect that many receptor types are necessary to cover all perceivable stimuli. Minimalistically, a glomerular organization may result from the need to have OSNs with the same response properties converging onto one target, thus glomeruli may be "just" the most parsimonious such organization.

Number, shape, and arrangement of glomeruli is a strongly species-specific property: adult *Drosophila* fruit fly has less than 50 glomeruli (Laissue et al. 1999; Stocker 1994), a moth (*Manduca*) has ~60 glomeruli (Rospars and Hildebrand 1992; Sanes and Hildebrand 1976c), a cockroach has ~125 glomeruli (Ernst et al. 1977), a worker honeybee ~160 (Flanagan and Mercer 1989a; Galizia et al. 1999a), and some ant species have over 200 (Rospars 1988) or even over 400 glomeruli (Zube et al. 2008). Glomeruli can be densely packed, as in *Drosophila*, or they can be arranged around a central area of the AL, the coarse neuropil. Individual glomeruli are not uniform: in bees the outer cap is the recipient of OSN input, while the core of each glomerulus is dominated by PNs. Some LNs branch in both the core and the cap of a glomerula, others do not, or only in some glomeruli. Serotonergic neurons innervate only the cap, dopaminergic neurons only the core of glomeruli in bees. The functional relevance of this glomerular subdivision into cap and core is as yet unknown. Furthermore, in immunostainings for protein kinase C, small circular regions of dense staining are visible within honeybee glomeruli (Grünbaum and Müller 1998). Glomerular subcompartmentalizations have been shown for *Drosophila* (Laissue et al. 1999), and are also known from vertebrates (Kasowski et al. 1999). Thus, there is an inner life to a glomerulus that remains to be elucidated.

The arrangement of glomeruli in locusts differs from that in most other species (Ignell et al. 2001). Each OSN axon innervates not one, but many glomeruli, and PNs also innervate many of the approximately 1000 glomeruli (Anton and Hansson 1996; Ernst et al. 1977). Even though the AL of locusts differs from other insects, functional properties may be common. For example, application of the chloride channel blocker, picrotoxin, has similar effects on the temporal response structure in PNs in bees (Stopfer et al. 1997) and in locusts (MacLeod and Laurent 1996).

The best knowledge of synaptic connections between neurons in the AL comes from ultrastructural studies in cockroaches (Distler and Boeckh 1996, 1997; Distler et al. 1998b; Malun 1991a, 1991b) (see also Figure 2.1B). Synaptic contacts occur between all neuron types: OSNs synapse onto LNs and onto PNs, and their presynaptic terminals receive input from LNs. LNs synapse onto OSN terminals, onto other LNs, and onto PNs. PNs get input from OSNs and from LNs, and they synapse onto other cells in the AL. The only contact that has not yet been shown is from PNs onto OSNs. Synapses are often dyadic, i.e., one presynaptic element makes contact with two postsynaptic elements, or reciprocal, or even more complex.

One of the major strengths of insects in neuroscience research is the notion of identifiable neurons. Similarly, olfactory glomeruli are also identifiable, and can be mapped from one animal to the next, always within one species, and sometimes for closely related species also. The characteristic arrangement, size, and shape of individual glomeruli helps in identifying glomeruli, and threedimensional atlases have now been created for a variety of species (Rospars 1988). Honeybee glomeruli are named according to the antennal tract that innervates them (T1–T4) and a number (i.e., T1-1, T1-2, or T3-33) (Flanagan and Mercer 1989a). An electronic atlas is available on the internet (Galizia et al. 1999a). *Drosophila* glomeruli are named with a letter or two, indicating the AL area, and a number, e.g., DM2 for dorso-medial-2 (Laissue et al. 1999; Stocker et al. 1990). Glomeruli that are identified later can be easily included into both nomenclatures (Couto et al. 2005; Kirschner et al. 2006). Atlases are also available for different moth species (Berg et al. 2002; Huetteroth and Schachtner 2005; Ignell et al. 2005; Masante-Roca et al. 2005; Rospars and Hildebrand 1992; Skiri et al. 2005).

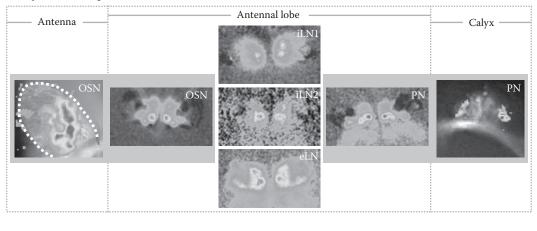
#### 2.2.5 GLOMERULAR ODOR RESPONSES

Stimulation with an odor leads to characteristic spatiotemporal glomerular activity patterns (Figure 2.2). Optical imaging of intracellular calcium concentration has been performed in bees (Galizia and Menzel 2001), in flies (Fiala et al. 2002; Wang et al. 2003), in moths (Carlsson et al. 2002; Galizia et al. 2000b; Skiri et al. 2004), and in ants (Galizia et al. 1999b). The conceptional similarity of these results in species other than *Drosophila* suggests that, also in these species, OSNs expressing the same OR are likely to converge generally onto a single glomerulus (an assertion that is commonly assumed, but not shown). Additional support comes from the observation that in the honeybee, *Apis mellifera*, the number of OR genes is close to 160, matching the number of glomeruli (Robertson and Wanner 2006). However, in the beetle, *Tribolium*, there are fewer glomeruli than the number of 341 receptor genes—more research is needed to investigate whether the same processing logic is implemented differently, or whether the logic itself differs (Engsontia et al. 2008).

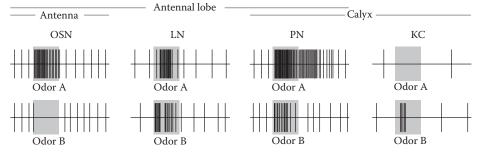
Some glomeruli are narrowly tuned (i.e., respond to a single or, at best, very few substances). Thus, activity in OSNs innervating this glomerules is sufficient information for an animal to behave assuming that the substance is in the environment. Such a specialized system would be able to code for only as many odors as there are glomeruli (which is approximately 43 in the adult *Drosophila*). Other glomeruli are broadly tuned, and create a combinatorial code. Here, the information carried by individual glomeruli is ambiguous, but by a comparison across glomeruli, odor information becomes precise. Many OSNs are broadly tuned at high concentration, but may be narrowly tuned at concentrations several orders of magnitude lower (Pelz et al. 2004; Røstelien et al. 2000; Stensmyr et al. 2003). In their natural environment, animals experience odorants over a large range of concentrations. Thus, the low-concentration mode may be useful in some situations; here, even though only a single receptor may be active, the lack of activity in the remaining glomeruli would signal the low-concentration mode. Thus, even the low-concentration coding scheme has a combinatorial logic.

Labeled lines are often mentioned in the context of sexual pheromones. However, in most cases, these are also combinatorial signals. Indeed, most pheromones consist of blends of several

#### (A) Optical recording



#### (B) Electrophysiology



**FIGURE 2.2** (See color insert following page 206.) Examples for odor-evoked activity in the fruit fly. (A) (*left*) Responses to the odor isoamyl acetate in OSNs on the antenna (their dendritic compartments) and in the antennal lobe (their axonal compartments) are shown left. The calcium sensor was expressed under Or83b control. (*Center*) Responses to the same odor in two inhibitory (iLN1, iLN2) and one excitatory (eLN) local neuron line within the AL. (*Right*) Responses to the same odor in projection neurons (line: GH146) within the antennal lobe (their dendritic compartments), and in the mushroom bodies (their axonal compartment). For the antenna and the mushroom body, nonactive areas were removed to visualize the underlying morphology. (B) Schematic view of characteristic activity patterns as spike trains, following the same sequence as in A, i.e., OSN, LN, PN and the mushroom body intrinsic KCs. Note the sparse firing properties of KCs as compared to PNs.

substances, which activate several glomeruli, and extraction of the correct pheromone-blend information is done by a combinatorial analysis of the respective glomerular activities (Christensen and Hildebrand 2002; Galizia et al. 2000b; Shirokova et al. 2005). Closely related species can thus use the same substances for their species-specific sex-pheromone signals, as long as they use them in a species-specific concentration ratio.

Most glomerular responses are temporally structured, and inherit this property from OSNs: some are inhibited by odors, some show rebound excitation at the end of the stimulus, some fire for a long time irrespective of stimulus duration, others only fire for a very short time, and stop even if the stimulus continues (de Bruyne et al. 2001). This is in addition to the intrinsic temporal complexity that airborne odors always have (Murlis et al. 1992; Vetter et al. 2006). As a consequence, odor-evoked activity patterns are already temporally complex at the input level of the olfactory system. These temporal patterns are further shaped by neural networks within the ALs (see Section 2.2.14). Possibly, these temporal structures are used for odor analysis.

#### 2.2.6 OLFACTORY SENSORY NEURON (OSN) TRANSMITTERS

The neurotransmitter used by OSNs is generally believed to be acetylcholine (ACh), which is the primary excitatory transmitter in the insect brain. The evidence is mostly based on the detection of the ACh-synthesizing enzyme, choline acetyltransferase (ChAT) (Bicker 1999b; Kreissl and Bicker 1989). ACh is present in *Manduca* OSNs (Sanes and Hildebrand 1976a; Stengl et al. 1990). Pressure application of ACh leads to activity (depolarization and hyperpolarization) in moth AL neurons, mediated by nicotinic receptors (Waldrop and Hildebrand 1989). In *Drosophila*, a ChAT/lacZ tranformant labels OSNs (Yasuyama et al. 1995), but immunoreactivity for ACh is low (Yasuyama and Salvaterra 1999). In all species studied so far, however, labeling was heterogeneous among glomeruli. Therefore, there might be another, as yet unknown, transmitter (or cotransmitter) in OSNs (Homberg et al. 1995; Homberg and Müller 1999; Kreissl and Bicker 1989), or glomeruli might differ in receptor and/or vesicle density.

An important neuronal messenger is nitric oxide (NO). The NO/cGMP system is found in insect' ALs, but the cells involved may not always be the same. In moths, NO is released by OSNs (Gibson and Nighorn 2000), and is important for proper glomerular development in the AL (Gibson et al. 2001). In cockroaches and locusts, however, NO is not synthesized in LNs (Elphick et al. 1995; Ott and Elphick 2002; Seidel and Bicker 1997). The NO source is unknown for bees, but NO is involved in olfactory habituation, and blocking NO disrupts olfactory discrimination (Bicker 2001; Hosler et al. 2000; Müller and Hildebrandt 2002).

#### 2.2.7 LOCAL NEURONS(LNS)

There are ~4000 LNs in bees (Witthöft 1967), ~360 LNs in moths (*Manduca sexta*) (Homberg et al. 1988), ~100 (GABAergic) LNs in *Drosophila* (Ng et al. 2002), and ~300 LNs both in cockroaches and locusts (Anton and Homberg 1999).

There are several different LN types in insects. Two classes are distinguished on morphological grounds: one type innervates most if not all glomeruli uniformly (homoLNs), the other innervates only a few (asymmetricLN) (Anton and Homberg 1999; Ernst and Boeckh 1983; Flanagan and Mercer 1989b; Matsumoto and Hildebrand 1981; Sun et al. 1993). Other "local" neurons include a population that innervates some dorsal glomeruli of the AL and areas of the dorsal lobe (Flanagan and Mercer 1989b). Bilateral neurons connecting both ALs have been found in bees (Arnold et al. 1985; Mobbs 1985) and flies (Stocker 1994; Stocker et al. 1990). In flies, neurons differ in their morphology and the innervated glomeruli, with several genetic differences that allow for the generation of specific markers of LN groups (Das et al. 2008). Both GABAergic (inhibitory) and cholinergic (exhitatory) neurons have been characterized (Shang et al. 2007; Silbering et al. 2008).

GABA-like immunoreactivity in LNs has been shown in bees (Schäfer and Bicker 1986), moths (Hoskins et al. 1986), *Drosophila* (Buchner 1991; Jackson et al. 1990), cockroaches (Distler et al. 1998b; Malun 1991b), and locusts (Ignell et al. 2001; Leitch and Laurent 1996). In bees, there is a small population of histaminergic LNs (Bornhauser and Meyer 1997), which likely acts as an inhibitory transmitter (Sachse and Galizia 2002). Since OSNs in flies express G-protein-coupled glutamate receptors, there probably is a population of glutamatergic LNs that synapse onto (at least some) OSN terminals (Ramaekers et al. 2001). In addition, there are cholinergic LNs (Shang et al. 2007).

LNs often express or coexpress peptides. Among these, allatotropin, allatostatins, tachykinins, FMRF-amide, and other RF-amide peptides have been found. The patterns of peptide antiserum staining differ widely among the species (Davis et al. 1996; Homberg 2002; Homberg et al. 1990; Homberg and Müller 1999; Iwano and Kanzaki 2005; Nässel 1993, 2000; Schachtner et al. 2004). Some neuropeptides are coexpressed with GABA, while others are not.

LNs have sodium action potential in honeybees (Galizia and Kimmerle 2004; Sun et al. 1993), moths (Christensen et al. 1993), and flies (Wilson et al. 2004). In locusts, only nonspiking LNs have been reported so far (Laurent and Davidowitz 1994). In cockroaches, in addition to spiking LNs

(Ernst and Boeckh 1983), there is a population of LNs without voltage-dependent sodium channels, and strong intracellular calcium activity (Husch et al. 2009). In bees and moths, intracellular recordings often show multiple spike amplitudes, suggesting either the presence of multiple spike initiation zones, or that LNs are electrically coupled. In some cases, artificial electrical connections created by the penetrating sharp electrode may create multiple spike heights (Galizia and Kimmerle 2004). Asymmetric LNs in honeybees have distinct odor-response profiles and respond to the odors associated with their main glomerulus (Galizia and Kimmerle 2004). In contrast, homoLNs studied in *Drosophila* have very broad response profiles, responding to most odors, with activity spread across large parts of arborization (Ng et al. 2002; Silbering et al. 2008; Wilson et al. 2004).

#### 2.2.8 **PROJECTION NEURONS (PNs)**

In honeybees, the IACT and mACT count about 400 fibers each, the mIACT is smaller, giving a total of less than 1000 PNs altogether (Rybak 1994). PNs in bees have also been estimated at 500 (Bicker et al. 1993) or 800 (Hammer 1997). The total number of PNs in *Drosophila* is estimated to be 150–200 (Stocker et al. 1997). The proportion of multiglomerular PNs with respect to uniglomerular PNs is below 10% in *Drosophila*.

Uniglomerular PNs branch in a single glomerulus within the AL and innervate both the mushroom bodies (MBs) and the lateral protocerebrum (LP). In most species, uniglomerular PNs form two distinct tracts, one located close to the brain midline (mACT in bees, iACT in other species), and the other traveling laterally (IACT). Multiglomerular neurons branch in several, if not all glomeruli, and generally do not innervate the MBs, but a variety of other areas in the protocerebrum. Generally, multiglomerular PNs use a group of smaller, intermediate tracts. PNs have both input and output synapses within olfactory glomeruli.

In bees, the two uniglomerular PN tracts, IACT and mACT (medial ACT) (Mobbs 1982), innervate a distinct AL hemilobe each. Interestingly, each hemilobe is also innervated by different tracts of the antennal (sensory) nerve: IACT glomeruli are innervated by T1, mACT glomeruli are innervated by T2–T4 (Abel et al. 2001; Bicker et al. 1993; Kirschner et al. 2006). In Manduca, tracts are iACT (inner, with uniglomerular PNs), mACT (medial, multiglomerular PNs, do not innervate the MBs), and oACT (outer, multiglomerular PNs, innervate the MB calyx) (Homberg et al. 1988). Fly tracts are named inner tract (iACT, traveling along the brain midline), which forms the strongest input to the MB calyces, mACT and inner-medial tract (imACT), which have some axons that innervate the MB calyces, while most axons target only the LP, and outer tract (oACT), which does not innervate the MB calyces (Figure 2.1C). All tracts innervate the LP (Stocker 1994; Stocker et al. 1990; Strausfeld et al. 2003). The GH146 line labels PNs with somata in the anterodorsal cluster (adPN) and lateral cluster (IPN), which have axons that use the iACT and send their dendrites to about 30-35 stereotypical glomeruli (Jefferis et al. 2001; Marin et al. 2002; Wong et al. 2002). GH146 also labels at least four PNs that use the mACT and at least one PN that uses the oACT. PNs from a given glomerulus always branch in the same MB target area (Jefferis et al. 2007). The POU domain transcription factors, Acj6 and Drifter, are expressed in adPNs and IPNs, respectively, and are required for their dendritic targeting (Komiyama et al. 2003). Interestingly, these genes are also necessary for OSN targeting (see above).

The situation in locusts is clearly different from that in other insects. In locusts, only a single ACT close to the brain midline connects the ALs to the MB (Leitch and Laurent 1996), and a few minor tracts connect the AL to other areas (Ignell et al. 2001). PNs are not uniglomerular, but branch in a limited number of glomeruli within the AL (Anton et al. 2002; Laurent et al. 1996). This is reminiscent of the multiple glomeruli innervated by individual OSNs in this species, but the two do not form corresponding groups, i.e., PNs innervate groups of glomeruli that do not correspond to groups innervated by an OSN (Anton et al. 2002). Therefore, glomerular groups in locusts cannot be functionally analogous to single glomeruli in other insects.

Many, but possibly not all, uniglomerular PNs have ACh as their transmitter. In bees, mACT PNs are AChE positive, showing that they use ACh (Kreissl and Bicker 1989). Furthermore, Kenyon cells (KCs) express nicotinic ACh receptors, and are activated by ACh (Bicker and Kreissl 1994). In moths, ~67 somata associated with the AL stain for AChE, and the outer ACT leading to the LP and the MB is stained (Homberg et al. 1995). The situation in *Drosophila* is similar, with at least one tract strongly stained for AChE (Yasuyama and Salvaterra 1999). In locusts, the ACT tract stains for AChE (Homberg 2002). However, whether the remaining PNs are also cholinergic and do not stain for some biochemical reason, or whether they use another—as yet unknown—transmitter remains to be elucidated.

GABAergic multiglomerular PNs have been shown in bees (Schäfer and Bicker 1986), moths (Hoskins et al. 1986), and flies (K. Ito pers. comm.).

Responses in insect PNs have been measured at the single cell level with electrophysiological techniques in a variety of species (Abel et al. 2001; Christensen et al. 1998a, 1998b; Galizia and Kimmerle 2004; Hansson et al. 1991; Müller et al. 2002; Wilson and Laurent 2005), including fruit flies (Bhandawat et al. 2007; Olsen and Wilson 2008; Schlief and Wilson 2007). Optical methods were successful in bees after loading PNs with calcium-sensitive dyes (Sachse and Galizia 2002, 2003), and in flies after genetically expressing activity-sensitive proteins (Fiala et al. 2002; Ng et al. 2002; Silbering and Galizia 2007; Silbering et al. 2008; Wang et al. 2003; Yu et al. 2004) (Figure 2.2). PNs are spontaneously active in insects, including honeybees (Abel et al. 2001; Galán et al. 2006), moths (Christensen et al. 1998b), *Drosophila* (Wilson et al. 2004), cockroaches (Boeckh et al. 1987), and locusts (Perez-Orive et al. 2002). Against this activity background, responses to odors can be both excitatory and inhibitory. PN responses are shaped by inhibitory networks within the AL (Bhandawat et al. 2007; Christensen et al. 1998a; Olsen and Wilson 2008; Sachse and Galizia 2002; Schlief and Wilson 2007; Silbering and Galizia 2007; Silbering et al. 2008; Wilson and Laurent 2005).

Instantaneous response frequencies of excitatory responses can be several hundreds of Hertz, and persist over the duration of a 2-s stimulus.

#### 2.2.9 FEEDBACK NEURONS AND BIOGENIC AMINES

Insect brains have characteristic, very large neurons that stain with antibodies against biogenic amines (dopamine, serotonin, octopamine, histamine) and innervate large areas of the brain (Bicker 1999a; Homberg 2002; Monastirioti 1999; Nässel 1999; Pflüger and Stevenson 2005; Roeder 1999; Stevenson and Sporhase-Eichmann 1995). These neurons are believed to have a modulatory function, including up and down regulation, thresholding, motivational states, attention, and learning (Bicker 1999a; Hammer and Menzel 1998; Homberg and Müller 1999). Thus, they also form part of a feedback channel from higher brain areas to the ALs. Other known feedback neurons include the honeybee AL-1 neurons, which originate in the MB  $\alpha$  lobe and project widely through the ALs (Rybak and Menzel 1993).

#### 2.2.10 GLIAL CELLS

Glial cells have been intensively studied in moths (Kretzschmar and Pflugfelder 2002; Oland and Tolbert 2003; Tolbert et al. 2004). Other species with information about glial cells are bees (Hähnlein and Bicker 1996), *Drosophila* (Awasaki et al. 2008; Jhaveri and Rodrigues 2002; Jhaveri et al. 2000, 2004), cockroaches (Prillinger 1981), and locust ALs (Hähnlein et al. 1996). The cells are prominent on the outside of the AL, and form thin processes that digitate between glomeruli, creating a boundary between them. In species where glomeruli are arranged around a central, non–glomerular neuropil (coarse neuropil, e.g., honeybees, moths), glial cells do not form a border between glomeruli and the central neuropil.

There are several different types of glial cells. Along the antennal nerve, they enwrap axon fascicles with long processes and multiple expansions. OSN axons travel as parallel bundles within

the antennal nerve, but as they approach the AL, they reach a so-called "sorting zone," where they form a dense and complex network and rearrange, in order to target the correct glomeruli, a process that necessitates functional glial cells (Oland et al. 1988; Rössler et al. 1999). The distinct nerves that innervate the AL (e.g., T1–T4 in bees) do not correspond to the distinct branches of the antennal nerve within the antenna (dorsal and ventral nerve). Within the AL, there are at least two glial cell types that form borders around glomeruli: one has large cell bodies and branching, vellate arbors. The other has multiple, mostly unbranched processes with many lamellate expansions along their length, which surround glomeruli as part of a multilamellar envelope (Oland et al. 1999). Glial cells are needed to form protoglomeruli and for correct AL development (Baumann et al. 1996).

Functions that have been proposed for glial cells in the adult system include the control of ion diffusion across glomeruli, or the formation of NO sinks (Gibson et al. 2001; Goriely et al. 2002).

#### 2.2.11 Odor-Evoked Activity

Given the circuitry of AL neurons known so far, it is possible to attribute putative functions to different cell types (Sachse and Galizia 2006). Thus, homogeneous LNs might serve as a gain control mechanism (either as inhibitory neurons or as excitatory neurons or both), asymmetric LNs would compute glomerulus-specific information, multiglomerular PNs respond to global activity, and thus give information about stimulus timing (onset/offset) and odor concentration, while uniglomerular PNs encode odors in their combinatorial activity pattern across their axons' identities.

As a result of different odor-response profiles across receptor cells, an odor stimulus leads to a characteristic activity pattern across individual glomeruli (Figure 2.2). Importantly, these activity patterns are not binary (on/off), but continuous (each glomerulus can be activated to varying degrees). From these patterns, it is possible to identify the stimulating odor, and therefore it would be sufficient as an olfactory code. However, it is not known what information is really used by the brain, and therefore the biological olfactory code remains to be elucidated.

Odor-evoked combinatorial patterns can be measured with imaging techniques, which afford measuring many glomeruli at the same time. Such responses have been measured in *D. melanogaster* by using genetically encoded reporter proteins. synaptopHluorin was used to measure synaptic vesicle release (Ng et al. 2002; Yu et al. 2004) and cameleon or G-CaMP for intracellular calcium (Fiala et al. 2002; Suh et al. 2004; Wang et al. 2003; Silbering and Galizia 2007; Silbering et al. 2008). These proteins were expressed under the control of specific promoters, which allow for a good reproducibility of the measured cells. For example, using the GAL4-line, GH146, a reporter protein can be expressed in a population of brain cells that within the AL only consists of PNs. Similarly, expressing the reporter in subpopulations of LNs allows the dissection of their relative contribution to odor-evoked activity.

Apart from genetically encoded reporter proteins, a large number of synthetic reporters are available, and have been used, in particular, in species other than *Drosophila*. Such dyes can be washed into the brain, as done for calcium-sensitive dyes in bees (Galizia and Menzel 2001; Joerges et al. 1997), ants (Galizia et al. 1999b), and moths (Carlsson et al. 2002; Galizia et al. 2000b; Hansson et al. 2003), and for NO release in moths (Collmann et al. 2004). Here, NO activity patterns and calcium activity patterns were similar, as expected, given that in moths, NO is produced by OSNs (Gibson and Nighorn 2000). Odor responses can also be measured with voltage-sensitive dyes (Galizia et al. 1997, 2000a). Cell-specific measurements can be obtained by staining specific cell populations, as done for PNs (Sachse and Galizia 2002, 2003). These selective stainings lead to calcium responses that are fast, with a steep rise at stimulus onset and a steep decay at stimulus offset. As in mammals (Leon and Johnson 2003; Xu et al. 2000), the spatial odor-response patterns have also been recorded using 2-deoxyglucose labeling in fruit flies (Buchner and Rodrigues 1983; Rodrigues and Buchner 1984), and in *Calliphora* (Distler et al. 1998a). In insects, because olfactory glomeruli are few and often easily recognizable by their relative position, shape, and size, functional atlases have been created of glomerular responses

(e.g., http://neuro.unikonstanz.de/honeybeeALatlas). Using multielectrode arrays affords the simultaneous extracellular recording of many neurons, but the neurons involved cannot be identified (Christensen et al. 2000).

#### 2.2.12 CONTRAST ENHANCEMENT

Information from input neurons (OSN) is transformed by internal connections (LNs) into the activity of output neurons (PNs). Consequently, the AL has often been modeled as a prototypical neural network in computational neuroscience (Bazhenov et al. 2001; Getz 1991; Getz and Lutz 1999; Linster et al. 1994; Rabinovich et al. 2000). The question of what role the hidden layers play in such a network can easily be addressed by comparing the glomerular activity of PNs with the glomerular activity in the OSNs, which in this system is particularly elegant given that both have the same number of dimensions (i.e., the same number of glomeruli). This approach has been taken in several studies, with surprisingly disparate results. In some optical imaging studies in Drosophila, the difference between PNs and OSNs was so small that no apparent processing was deducible (Ng et al. 2002; Wang et al. 2003). In an electrophysiological study comparing input and output of glomerulus DM2, also in Drosophila, the response spectrum was apparently broader in PNs than in OSNs (Wilson et al. 2004). In another study in Drosophila, presynaptic inhibition onto receptor cell terminals led to an inhibitory network activity (Olsen and Wilson 2008). Comparing input and output for odor concentration dose-response curves in honeybees showed that glomeruli with a low threshold for an odor have almost identical dose-response curves in PNs and in OSNs, while less sensitive glomeruli have a shifted dose-response curve in PNs, showing that at higher concentrations, LNs suppress these PN responses (Sachse and Galizia 2003). Linster and colleagues created a computer model of the AL and ran experimental data under different assumptions about LN network connectivity, comparing three network architectures: one where LNs interconnect neighboring glomeruli, one with stochastic connections, and one based on the odor-response properties of glomeruli. The results show that inhibitory connections are strongest among glomeruli that have similar odor-response profiles, and weakest among glomeruli that do not overlap in their odor-response profiles, irrespective of their spatial position (Linster et al. 2005). This leads to an amelioration of odor responses in PNs across odors, by reducing response overlap to similar odors. Interestingly, the system need not be symmetrical: an inhibitory connection from glomerulus A to a glomerulus B does not imply a similar connection from B to A. Physiological evidence for such a nonsymmetrical connectivity has been found (Sachse and Galizia 2002), and behavioral experiments confirm such an asymmetry: similarity from an odor X to Y can be different from the similarity of Y to X (Guerrieri et al. 2005).

#### 2.2.13 SENSITIVITY OPTIMIZATION IN THE ANTENNAL LOBE (AL)

PNs are spontaneously active with a pronounced temporal complexity, resulting in continuously changing, low-level glomerular activity patterns, even in the absence of sensory stimulation (Galán et al. 2006; Sachse and Galizia 2002). If the response magnitude were normalized, it would be difficult or even impossible to distinguish individual events of spontaneous activity from odor-evoked responses. The driving force for this spontaneous activity might come from background activity in OSNs. Within the AL network, background activity can be tightly controlled to be kept just at threshold, a scenario that would increase the sensitivity to weak odors (Sachse and Galizia 2006; Shang et al. 2007). Conceptionally, PNs can be compared to a loaded spring. A tight regulation of inhibitory and excitatory LNs keeps PNs at firing threshold, so that a minimal olfactory stimulus would already elicit an odor-evoked pattern. PNs are maintained close to threshold by constantly probing their depolarization, which results in a level of spontaneous activity. Even though this system leads to a continuous shift of the baseline, odor–concentration coding is not affected, because the odor–concentration magnitude of a stimulus remains available

in the first derivative of the odor response (i.e., in the steepness of the response). Odor concentration information is much more affected by receptor adaptation at the periphery, a phenomenon known from all sensory systems. As with most sensory systems, the olfactory system is better at measuring concentration changes rather than absolute concentrations. The "loaded spring model" of the AL ensures that small increases in odor concentration will lead to a strong response, even when background odors are present.

#### 2.2.14 TEMPORAL ACTIVITY STRUCTURES

In the natural environment, odors are temporally complex due to air turbulence (Justus et al. 2005; Murlis et al. 1992), and temporal odor-response patterns predominantly reflect stimulus variation (Vickers et al. 2001). In addition, OSNs already have temporally complex response properties: some have phasic, some tonic responses, some have activity that outlasts the odor stimulus, and some reduce activity upon olfactory stimulation (de Bruyne et al. 2001). As a consequence, even an olfactory stimulus that is temporally uniform leads to a temporally complex pattern of activity (see also Chapters 12 and 13).

Slow temporal structures consisting of sequences of bursts and inhibitory events have been observed in PNs of all insects studied so far, including moths (Christensen et al. 1998b; see also Chapter 3), locusts (Laurent 1996), and honeybees (Abel et al. 2001; Müller and Hildebrandt 2002).

Behavioral studies have shown that olfactory discrimination is in the range of ~200 ms in rats (Abraham et al. 2004; Uchida and Mainen 2003) and ~690 ms in bees (Ditzen et al. 2003), a time that includes the time needed for the motor responses and physical displacement of the animal in that particular task. Odor similarity or odor concentration have no or only a small effect on the time needed for olfactory discrimination (Abraham et al. 2004; Ditzen et al. 2003; Uchida and Mainen 2003). Physiological studies of PN responses show that 200–300 ms in locusts (Stopfer et al. 2003) and maximally 400 ms in bees (Galán et al. 2004) are needed to reach the most distinct odor classification in the AL. Therefore, all phases of slow activity components after this time are irrelevant for odor discrimination. However, late activities might be important for olfactory learning or other aspects of olfactory processing; in fact, odor representation is ameliorated during the first 2 s after stimulus onset, leading to a clearer distinction of odors (Friedrich and Laurent 2001; Galizia et al. 200a).

Fast temporal structures are evident in odor-evoked oscillations, which are found almost ubiquitously in olfactory systems. Within insects, oscillations have been shown in cockroaches, locusts, bees, wasps, flies, and moths (Heinbockel et al. 1998; Stopfer et al. 1999). The chloride channel blocker, picrotoxin, abolishes these oscillations (MacLeod and Laurent 1996; Stopfer et al. 1997), but also modifies combinatorial spatial activity patterns (Sachse and Galizia 2002). Individual PNs do not fire in every oscillation cycle, and action potentials also occur out of the synchrony pattern. Therefore, odor identity could be encoded in sequences of changing PN ensembles (Laurent 1999). Alternatively, or in addition, synchrony could be related to odor concentration or intermittency rather than odor quality (Christensen et al. 2000).

#### 2.2.15 COMBINATORIAL ODOR CODES

Odors evoke combinatorial patterns of activated glomeruli, with each glomerulus participating in the activity patterns of many odors. These patterns are conserved among individuals (Galizia et al. 1999c; Wang et al. 2003), which is a consequence of the innate mapping of OSNs that express a given OR to individual glomeruli (Couto et al. 2005; Fishilevich and Vosshall 2005; Vosshall et al. 2000). Molecular receptive ranges (MRR) are best described by the response range to many odors (Sachse et al. 1999), as is the case for the receptors themselves (Hallem and Carlson 2006; Pelz et al. 2006). There are no glomeruli for functional groups or other chemical parameters (e.g., "aldehyde" or "C6-carbon-chain"). Therefore, the response profile of individual glomeruli is not determined

by particular "features" of the odorant (sometimes referred to as odotopes), such as "ketone group" or "aldehyde," and the olfactory code is not a building set, where 1-heptanol would be coded in an "alcohol glomerulus" plus a "C7 aliphatic chain" glomerulus.

#### 2.2.16 ODOR CONCENTRATION AND MIXTURES

With increasing odor concentration, responses increase both in magnitude and in number of active glomeruli. Similarly, with decreasing concentration, activity decreases, and may consist of a single glomerulus being active at very low concentration. This is a direct consequence of the receptors' response properties: each receptor has a few substances to which it responds with higher affinity than any other receptor, and at its lowest effective concentration, that substance will elicit a combinatorial pattern of activity in the AL, which consists of that single glomerulus being active, and all other glomeruli being silent. PN responses in honeybees are qualitatively stable over a concentration range of up to 4 log units, because higher odor concentrations increase total response intensity without changing the relative intensity across glomeruli; at the input level, however, the activity patterns are more affected by concentration differences (Sachse and Galizia 2003). Thus, the neural network within the AL contributes to concentration invariance. Comparable results were found in *Drosophila* (Silbering et al. 2008).

When an odor consists of many substances, the task for the organism might be either to recognize that particular mixture (e.g., the characteristic bouquet of coffee), or to extract a component (e.g., the presence of garlic in the food). Physiologically, the presence of an odor Y can interfere with the normally strong response to an odor X, which is termed mixture suppression. Conversely, synergism indicates when a neuron or glomerulus responds to a binary mixture with a response that exceeds the summed responses to the single components. An inhibitory network within the AL, optimized for sharpening odor-response patterns, should create stronger mixture interactions when similar odors are mixed than when dissimilar odors are mixed. Psychophysically, similar odors in a mixture "compose" a new odor, which makes it difficult for the olfactory system to extract the identity of the odor components (synthetic representation), while mixtures of dissimilar substances are represented as the sum of the optimized representation of each component (analytical representation) (Wilson and Stevenson 2003). Behavioral data in rats support this idea (Wiltrout et al. 2003). By increasing the number of components in an odor mixture, mixture interactions increase and further reduce the similarity to the single component patterns (Deisig et al. 2003; Silbering and Galizia 2007). With this coding strategy of odor mixtures, the olfactory system implements a logic that allows a unique representation of odor mixtures without saturating the olfactory code, at the expense of losing analytical capacity.

#### 2.2.17 Special Cases: Sexual Pheromones

Generally, OSNs responding to sexual pheromones are highly specific, and form labeled lines for each pheromone component. The labeled line property is dependent on the environment: if, in the natural environment of the animal, there are no other, alternative ligands, the receptor functions as a labeled line, even though a chemist's collection might find other effective substances. For example, in *M. sexta*, the sexual pheromone is a blend of two chemicals, the main component (E,Z)-10,12-hexadecadienal, and the secondary component (E,E,Z)-10,12,14-hexadecatrienal. A different and more stable molecule, (E,Z)-11,13-pentadecadienal, is a good mimic and is routinely used instead in physiological experiments (Christensen and Hildebrand 1997). Coding of sexual pheromones is combinatorial, because each component is necessary for identifying that the pheromone is the species-specific blend.

Sexual pheromones in *Drosophila* are not involved in long-distance navigation, but rather are part of a "close-encounter" olfactory display, which is detected by contact chemoreceptors. The

only candidate for a volatile pheromone is cis-vaccenyl acetate, though its precise behavioral significance remains to be elucidated (Amrein 2004; Costa 1989). OSNs sensitive to cis-vaccenyl acetate reside in T1 sensilla on the antenna (Xu et al. 2005).

#### 2.2.18 SPECIAL CASES: CARBON DIOXIDE

Most arthropods have  $CO_2$  sensitive systems with a wide variability in structure and function (Bogner et al. 1986; Kleineidam and Tautz 1996; Stange and Stowe 1999). The sensilla can be hairs, pegs, plugged or open grooves, they can be on the surface, or they can be located within a depression or a pit with a restricted opening (Keil 1996; Stange and Stowe 1999). The relevance of  $CO_2$  also differs across species. In blood-sucking insects such as mosquitoes,  $CO_2$  sensitivity is often relevant for finding the host (Dekker et al. 2002; Grant et al. 1995). For nocturnal moths feeding on nectar,  $CO_2$  might be a component of the attractive flower odor, since flowers release considerable amounts of metabolic  $CO_2$  (Guerenstein et al. 2004; Raguso 2004; Thom et al. 2004). Insects that live in confined spaces, such as centipedes or beetle larvae, sense  $CO_2$  to ensure sufficient respiration. Similarly, social insects (ants, bees, and termites) monitor  $CO_2$  in their hives and control its concentration (Lacher 1964; Stange and Stowe 1999; Weidenmüller et al. 2002).

*Drosophila* is repelled by  $CO_2$ , and a role of this gas as a component of a stress signal has been suggested (Suh et al. 2004). However,  $CO_2$  is also produced by rotting fruit, and by fly aggregations on such fruit that might indicate good ovipositioning sites. In the *Drosophila*, AL  $CO_2$  activates the V glomerulus, suggesting a labeled-line-like system for this substance (Suh et al. 2004).

#### 2.3 THE MUSHROOM BODIES (MBs)

MBs are multimodal structures in the insect protocerebrum. They are involved in learning (Davis 2004; Heisenberg 2003; Strausfeld and Gilbert 1992), and receive both olfactory and visual input in most insect species (Farris 2005; Strausfeld et al. 1998). Their names derive from the massive peduncles with large, cup-shaped protuberances, which are called the calyces. In hymenoptera, the calyces are subdivided into lip, collar, and basal ring, which correspond to three separate bands in the  $\alpha$  lobe (or vertical lobe). In *Drosophila*, subdivisions from the calyces can be traced to a concentrically circular arrangement in the  $\alpha/\beta$  lobe and to a layered structure in the  $\alpha'/\beta'$  lobes (Tanaka et al. 2004). The peduncles generally branch into two lobes, the vertical ( $\alpha$ ) lobe and the horizontal ( $\beta$ ) lobe. In addition, in *Drosophila*, they form the  $\alpha'$  and the  $\beta'$  lobes (Strausfeld et al. 2003). A third lobe, the  $\gamma$  lobe, is physically attached to the vertical lobe (the  $\alpha$  lobe) in honeybees, but is morphologically distinct in *Drosophila* (Farris et al. 2004; Strausfeld 2002). The intrinsic neurons in the MBs are called KCs.

In the olfactory pathway, uniglomerular PNs form the input to MB calyces. In hymenoptera, this input is targeted at the lip and the basal ring of the calyces, while the intermediate area, the collar, receives input from the optic lobes (Gronenberg 1999, 2001). In *Drosophila*, each PN axon travels over large areas of the calyx, forming synapses with many intrinsic KCs (Marin et al. 2002; Wong et al. 2002). This is reminiscent of the situation in the mammalian olfactory cortex (Zou et al. 2005), and allows for a combinatorial readout of PN response patterns. PNs within the MB calyx region occupy concentric layers (Tanaka et al. 2004). PNs from identified glomeruli branch in a stereotypical manner within the MB calyces, and segregate in a functionally dictated way. For example, fruit odors and pheromone odors target other areas (Jefferis et al. 2007). On average, three uniglomerular PNs innervate each glomerulus, and these have the same projection pattern in MB and LP, suggesting that they are not functionally distinct (Wong et al. 2002).

Schematically, MB input can be described as a scaffold, with arrays of PN axons crossing arrays of KCs, and forming synapses with some, but not all KCs (Heisenberg 2003). In neural network language, this is ideal for combinatorial readout across PNs.

KC numbers differs widely among species: in honeybees, there are ~170,000 KCs in each hemisphere (Mobbs 1982; Witthöft 1967), *Drosophila* counts give ~2500 KCs (Stocker 1994). In adult cockroaches, the number is ~175,000 (Neder 1959), but juveniles have much smaller numbers (Farris and Strausfeld 2001), adult locusts have ~50,000 KCs (Farivar 2005). KC somata lie close to the MB calyces, in a densely packed manner. Their axons are long and thin and form the peduncles. The  $\gamma$  lobe is formed by the axons of the clawed KCs, the first subpopulation of KCs to occur in development (Farris et al. 2004; Mobbs 1982; Rybak and Menzel 1993; Strausfeld 2002). The name "clawed" derives from their claw-like dendritic shapes within the calyces.

KC morphology, pharmacology, and peptide expression shows a considerable variability across all species studied so far (Iwasaki et al. 1999; Sinakevitch et al. 2001; Strausfeld 2002; Strausfeld et al. 2000, 2003; Strausfeld and Li 1999a, 1999b). Glutamate labels a KC subpopulation of bees (Bicker et al. 1988). Aspartate, glutamate, and taurine immunocytochemistry label different KC populations in *Drosophila* (Strausfeld et al. 2003) and in cockroaches (Sinakevitch et al. 2001). In addition, in *Drosophila*, KCs produce NO (Schürmann 2000), but ACh and GABA are excluded as KC transmitters (Yusuyama et al. 2002).

#### 2.3.1 LOCAL INHIBITORY AND MODULATORY NEURONS

Synaptic arrangements in the MBs form microglomeruli with very local computational capabilities. In *Drosophila*, each MB microglomerulus comprises a large cholinergic bouton formed by a PN axon from the AL, which is surrounded by tiny vesicle-free KC dendrites and several GABAergic terminals (Yusuyama et al. 2002). GABAergic terminals contact both KC dendrites and PN axon terminals, suggesting that PN input is modulated both pre and postsynaptically (Ganeshina and Menzel 2001; Leitch and Laurent 1996; Yusuyama et al. 2002). MB microglomeruli have no glial sheath (Ganeshina and Menzel 2001; Yusuyama et al. 2002).

In addition to microglomerular circuits, there are GABAergic feedback neurons from the MB lobes back onto their calyces. These neurons are few in number, in honeybees ~55. Each feedback neuron innervates a subcompartment in the calyx, with each subcompartment in the calyx being connected to its specific, corresponding layer in the  $\alpha$  lobe (Grünewald 1999). These neurons have been found in bees (Bicker et al. 1985; Schäfer and Bicker 1986), moths (Homberg and Hildebrand 1994), *Drosophila* (Yusuyama et al. 2002), cockroaches (Farris and Strausfeld 2001), and locusts (Leitch and Laurent 1996).

Octopaminergic cellular processes sparsely but uniformly innervate MB calyces (Strausfeld et al. 2003). In honeybees, most of these processes are formed by the VUMmx1 neuron that represents the conditioned stimulus (CS) during olfactory learning (Hammer 1997). VUMmx1 also branches in the AL, the LP, and the subesophageal ganglion (SEG).

#### 2.3.2 OUTPUT LOBE CIRCUITRY

The pathways from AL to MB, and the internal circuitry of the MB, have been studied in more detail than the MB output to other brain areas, notably the LP. Generally, the output is believed to target premotor areas, and the pathway OSN-AL-uniglomerularPN-MB-LP-premotor areas-motor neuron would run in parallel to the pathway OSN-AL-multiglomerularPNs-LP-premotor areas-motor neuron loop, which bypasses the MBs. Several subregions within the protocerebrum have been identified, including the superior medial protocerebrum, the inferior medial protocerebrum, and the superior LP (Ito et al. 1998; Tanaka et al. 2004). MB output neurons in honeybees branch unilaterally or bilaterally (Rybak and Menzel 1993). A prominent large neuron in honeybee brains is PE1, which is a single neuron in each brain hemisphere, and connects the  $\alpha$  lobe to the LP and the ring neuropil around the  $\alpha$  lobe (Brandt et al. 2005; Rybak and Menzel 1993). (Okada et al. 2007).

#### 2.3.3 Odor-Evoked Activity in the Mushroom Bodies (MBs)

As compared to PNs, KCs respond to odors with few spikes, if any (Stopfer et al. 2003): while PNs had a response probability of p=.64, KCs responded with p=.11 to a given odor set (Perez-Orive et al. 2002). Also, PNs respond with trains of spikes, but KCs respond with single or only very few spikes (Figure 2.2B). Similar results were found in flies and honeybees (Szyszka et al. 2005; Wang et al. 2004). Thus, odor representation in KCs is sparse in the sense of population sparseness (a low proportion of units active at any time) and in the sense of lifetime sparseness (few spikes in each neuron with narrow tuning) (Laurent 2002; Olshausen and Field 2004). This sparsening is a progressive feature of MB circuitry: activity trains arriving at the MB terminals are inhibited presynaptically by GABAergic glomerular microcircuits, so that only the first APs are likely to drive activity in KCs (Assisi et al. 2007; Szyszka et al. 2005). In addition, the inhibitory feedback loop from the MB output lobes onto the calyces further sharpens that response (Szyszka et al. 2005). This feedback also generates a global oscillatory rhythm (Perez-Orive et al. 2002), which, in turn, favors the extraction of synchronized APs from PNs (Perez-Orive et al. 2004).

#### 2.3.4 OLFACTORY CODING

If only the initial firing pattern leads to KC activity in each odor puff (Szyszka et al. 2005), then most APs that PNs generate in an odor response cannot contribute to odor-information decoding in the MBs. Are the remaining APs wasted? Such a waste would be quite inefficient, considering that producing APs is among the most energy-costly activities of the brain (Attwell and Laughlin 2001). However, the "surplus" spikes may be relevant outside the MB, e.g., within the AL and in the LP, where PNs have other output synapses.

The massive expansion from relatively few PNs to many KCs in insect olfactory systems has been likened to a support vector machine (Galán et al. 2004). With an integrating neuron at the PN output level that reads across PN activities, it is only possible to perform a limited classification, which statistically corresponds to a linear classification in a multidimensional space. However, when the same number of PNs are first combinatorially mapped onto a very large number of KCs, an integrating neuron that would read across these activities could extract much more complex pattern topologies. Thus, the large number of KCs allows for the computation of highly nonlinear classification schemes across PNs (Huerta et al. 2004). If temporal complexity is added to the code, the theoretical capacity of the system increases even further (Laurent et al. 2001).

#### 2.4 OLFACTORY MEMORY AND PLASTICITY

#### 2.4.1 THE ANTENNAL LOBE (AL)

Brains respond to experience with changed behavior, a process generally called learning. Here, associative and nonassociative forms of plasticity are differentiated, because they differ both conceptionally, behaviorally, and in the cellular processes involved. Associative paradigms include classical conditioning and operand conditioning. Among the nonassociative processes, habituation, and sensitization are the most important ones. There are several sites in the brain where learning induces cellular changes. In the olfactory system, the AL as the first olfactory neuropil is already involved in substantial experience-induced changes. Some of these changes in odor responses occur without being directly attributable to a particular form of behavioral change (so far). For example, repeated exposure to an odor leads to changes in odor responses in PNs: the number of APs is reduced, but their temporal precision is increased, which may mean that their coding is more efficient for later puffs (Stopfer and Laurent 1999). This sensory memory trace decays within 10–15 min after the last puff (Stopfer and Laurent 1999). In honeybees, a single odor exposure leads to a change in the ongoing spontaneous activity across PNs: a pattern corresponding to the experienced

odor reoccurs repeatedly during the next 1–2 min, showing that the AL network creates an ephimeral neural attractor for this pattern (Galán et al. 2006).

Application of sucrose (or water) to the honeybee antenna leads to olfactory sensitization. It elicits a transient increase of PKA activity in the AL, but odor stimulation alone does not (Hildebrandt and Müller 1995a, 1995b). This effect is mediated by octopamine, and reverts to baseline within 3 s (Hildebrandt and Müller 1995a).

In classical conditioning, an odor is associated with a punishment (e.g., electroshock, aversive, and learning) or a reward (e.g., sugar water, and appetitive learning). Aversive conditioning leads to changes in the fly AL, where PNs in some glomeruli change their odor responses for about 3 min after conditioning (Yu et al. 2004). Appetitive associative learning is also mediated by octopamine, which represents the unconditioned stimulus (US). In bees, the US can be replaced by octopamine injections into the AL (Hammer and Menzel 1998). Blocking octopaminergic transmission either by injecting an octopamine receptor antagonist (mianserin) or by injecting double-stranded octopamine receptor RNA into the AL, interferes both with odor memory acquisition and with odor memory recall. Consequently, we must assume that octopamine is important for memory consolidation but not only, and that octopaminergic neurons become reactivated during memory recall (Farooqui et al. 2003). In honeybees, short-term memory (STM) can be generated by single-trial conditioning, and long-term memory (LTM) by multiple conditioning trials during training. Multiple conditioning trials lead to an elevated PKA response in the AL, which is mediated by the NO/cGMP system. STM can be converted into LTM when PKA activity is artificially increased after single-trial learning, suggesting that it is not the presence of PKA, but its concentration that is important for generating LTM (Müller 2000). Differential conditioning (one odor was rewarded, the other not) leads to a modification of odor-response patterns in the AL in the time window of 5-15 min after conditioning, as shown with calcium imaging in bees (Faber et al. 1999), and with extracellular recordings in moths (Daly et al. 2004).

Other studies have not found changes in the AL that are attributable to olfactory associative memory. In *Drosophila*, the MBs have been shown not only to be necessary, but also to be sufficient for short-term learning of odors, leaving no space for a memory trace in the AL (Gerber et al. 2004b). In honeybees, the uniglomerular PNs from the lACT tract have very stable odor responses that are not affected by single-odor training or differential appetitive training (Peele 2005). These neurons might represent a processing channel that ensures reliable transfer of odor-related information to higher order brain centers, a hypothesis that remains to be investigated. Together, these currently available data show that odor learning occurs in the AL and affects spike timing and/or relative activities to different odors. These effects, however, only occur in very limited time windows, are limited to specific cell populations, and only represent a part of the memory trace.

#### 2.4.2 THE MUSHROOM BODIES (MBs)

MBs play an important role in olfactory memory, as already shown by experiments in which memory retrieval was impaired when the MBs were cooled in honeybees (Erber et al. 1980). Similarly, learning deficits are observed in *Drosophila* mutants where MB structure is altered (*MB deranged*, *mbd*, and *MB miniature*, *mbm*) (Heisenberg et al. 1985). Blocking synaptic activity or disrupting MB physiology also leads to memory deficits (Connolly et al. 1996; Dubnau et al. 2001; McGuire et al. 2001). MBs can be chemically ablated by applying hydroxyurea, a DNA-synthesis inhibitor, during the early proliferation phase of KCs. This leads to olfactory memory impairment in *Drosophila* (de Belle and Heisenberg 1994). In bees, partial ablation of MBs only impairs complex tasks with several odors, but not easy learning tasks (Komischke et al. 2005; Malun et al. 2002).

It should be noted, though, that MBs are not indispensable. There are many olfactory tasks that can be solved without MBs, and indeed, fly mutants that lack MBs are remarkably normal: they feed, lay eggs, are alert, court and copulate, are well oriented in space, and respond to odors

(Heisenberg 2003). Furthermore, MBs have many tasks beyond olfaction: they are also used for spatial memory and navigation without olfactory cues (Kwon et al. 2004; Mizunami et al. 1998; Strausfeld et al. 1998).

In honeybees, the VUMmx1 neuron that represents the appetitive reinforcer also innervates the MB calyces. Because the VUMmx1 neuron represents the US, it is probably involved in the necessary mechanism of coincidence detection, which means that for appetitive olfactory learning in honeybees, coincidence detection occurs in the MB input region (Menzel and Giurfa 2001). Optical imaging experiments show that a rewarded odor leads to increased calcium responses in the MB calyces as compared to before learning (Faber and Menzel 2001; Szyszka et al. 2008). Morphological changes are also observed in honeybee MBs after learning: worker bees that forage have KC dendrites with more branches than age-matched bees that do not forage, while the density of dendritic spines remained constant (Farris et al. 2001).

The best cellular analysis of olfactory memory traces in MBs comes from *Drosophila* (Davis 2004; Dubnau et al. 2003; Heisenberg 2003; Waddell and Quinn 2001). Memories can be categorized based on how long they last: STM decays within 1 h, middle-term memory (MTM) within 3 h. Anesthesia-resistant memory (ARM) and LTM are two forms of LTM that differ in their training procedures: ARM occurs after massed training, and the protein-synthesis-dependent LTM occurs after spaced training, i.e., a training protocol where individual learning events occur with longer intervals in between (Tully et al. 1994). However, even though the nomenclature of this classification is based on time, the real classification should be based on the biochemical pathways associated with STM, MTM, ARM, and LTM.

KCs are the cells where associative olfactory memory is located in flies (Gerber et al. 2004b). Mutants for the genes *dunce (dnc,* which is a cAMP phosphodiesterase), DC0 (which is a PKA catalytic subunit), or CREB (cAMP response element binding protein) are impaired in STM tasks, showing that a necessary second messenger in STM is cAMP. The *Drosophila* gene *rutabaga (rut)* codes for a Ca/CaM-dependent adenylyl cyclase (Levin et al. 1992). *rut* mutants are olfactory learning defective, and expressing *rut* in MB cells restores learning, showing that MB cells are sufficient for learning (Zars et al. 2000). Several different *Drosophila* lines were used in these experiments that all differed in exactly which cells were restored. The cell population common to all successful rescue groups were the clawed KCs, suggesting that these are *sufficient* for short-term olfactory learning (Connolly et al. 1996). *rut* is only necessary in adult animals, but not during development (Mao et al. 2004; McGuire et al. 2003). *rut* acts presynaptically at the output synapses of KCs, and blocking synaptic release of these cells impairs retrieval, but not acquisition (Dubnau et al. 2001; Schwaerzel et al. 2002).

Thus, at first sight, this appears to be a difference between flies and bees (see above): in flies, the learning site is at the output synapse of KCs, while in bees it is at the input site. Possibly, this finding is not a species-related difference, but rather a task-related difference: most experiments in flies are made using aversive conditioning (with electric shock as US), most experiments in bees are made using appetitive conditioning (with food reward as US). Indeed, when appetitive conditioning is performed in flies, both PNs and KCs are independently sufficient for successful memory performance (Thum et al. 2007). The neuronal pathways for appetitive and aversive conditioning are clearly different, in particular for US representation. Therefore, it is not surprising that coincidence detection may occur in different places. In honeybees, appetitive learning is mediated by octopamine (Hammer and Menzel 1998), consistent with findings in *Drosophila*, while the aversive US is mediated by dopaminergic neurons (Schwaerzel et al. 2003).

The gene *amnesiac* (*amn*) is strongly expressed in the dorsal paired medial (DPM) neurons, of which there are two in each fly. *amn* codes for a neuropeptide (PACAP) that modulates *rut* activity in KCs. Disruption of *amn* or silencing of DPM neurons leads to loss of MTM. The activity of DPM is necessary at different times for some odors, but not for others. For the odors octanol and methyl-cyclohexanol, DPM neuron activity is necessary during storage and possibly consolidation, but not

during acquisition and recall, while memories for benzaldehyde need DPM neuron activity during acquisition (Keene et al. 2004). These data show that not only the cells and the neural networks have a high diversity in memory research, but even the odors used affect which cell, memory phases, genetic, and neural networks are used and relevant. Furthermore, the entire network needs to be functioning. Even if the engram was at a single synapse, without the network, memory would not be formed. For example, inhibitory MB output/feedback neurons are necessary for memory formation, and when they are silenced, no memory is formed (Liu et al. 2009).

LTM includes ARM and LTM. These are not sequential: ARM is formed even in *amn* mutants that show no MTM, and at least partially also in *rut* mutants that show no STM (Isabel et al. 2004). Different KC populations are needed for STM/MTM ( $\gamma$  lobe) and ARM/LTM ( $\alpha/\beta$  lobe) (Isabel et al. 2004; Pascual and Preat 2001; Zars et al. 2000). The ubiquitin ligase, Neuralized, is only used in  $\alpha/\beta$  lobe and is necessary for LTM, but not for ARM (Pavlopoulos et al. 2008). Nevertheless, LTM and ARM are not entirely independent pathways: rather, LTM induces an active erasure of ARM memory (Isabel et al. 2004). The cyclic AMP response element CREB appears to be related to LTM (Yin and Tully 1996), though the scientific evidence has recently been questioned (Perazzona et al. 2004).

## 2.4.3 ACTIVITY-DEPENDENT PLASTICITY

The ability of the brain to adapt structurally and functionally in response to sensory experience is a striking property across animal phyla. However, several studies have shown that the olfactory system of *Drosophila* possesses a remarkable wiring stability. Ablation of the olfactory input to the AL by cutting the antennae in the adult fly has no effect on dendritic or axonal arborization of the ascending PNs (Berdnik et al. 2006; Tanaka et al. 2004; Wong et al. 2002). The adult olfactory circuit seems to respect the glomerular boundaries imposed by the rules of development.

Despite this apparent wiring rigidity, the olfactory circuit appears to have the capacity for experience-dependent plasticity within the confines of a glomerulus. In honeybee workers, the volume of identified olfactory glomeruli is modified during adult life dependent on the behavioral task (Winnington et al. 1996). Studies from Drosophila show that deprivation of the input from one antenna reveals the existence of activity-dependent competition in the extent of axonal arborization (Berdnik et al. 2006). Moreover, continuous exposure of adult flies to single odors for several days causes either a stimulus-dependent decrease of the nonactivated, possibly inhibited glomeruli (Devaud et al. 2001, 2003), or results in a drastic volume increase of glomeruli activated by the stimulus used for long-term exposure (Sachse et al. 2007). Interestingly, chronic olfactory exposure does not affect the morphology or function of the OSNs, while one class of inhibitory LNs and the output of the PNs are functionally modulated in response to the exposed odor (Sachse et al. 2007). Longterm odor exposure has also been shown to affect the odor-guided behavior of different insects. The spontaneous aversive tendency of *Drosophila* to some specific odors can be reduced by exposing adult flies to these odors beforehand (Hershberger and Smith 1967). Studies in honeybees have shown that bees prefer an odor when they have previously associated that odor to a sugar reward, but not if they have just been exposed to that odor without reinforcement (Sandoz et al. 2000).

#### 2.5 THE LARVAL SYSTEM

In this chapter, we have only looked at the adult olfactory system in insects, and not at the maggot, for which there are several recent reviews that can be accessed (Cobb 1999; Heimbeck et al. 1999; Kreher et al. 2005; Marin et al. 2005; Scherer et al. 2003; Stocker 2001; Gerber and Stocker 2007). The main difference between the two systems is the greater simplicity in larvae, both in terms of cell numbers and organization, even though important principles are shared (Python and Stocker 2002a, 2002b). Odor perception is good in larvae, and odor learning is robust (Gerber et al. 2004a; Hendel et al. 2005; Scherer et al. 2003). The main pathway, OSN-PN-KC, is also realized in larvae,

but the main difference is that—at least in *Drosophila*—each glomerulus only receives input from a single OSN axon, and is innervated only by a single PN (Kreher et al. 2005; Ramaekers et al. 2005). The larval MBs also share several features of their adult counterparts (Marin et al. 2005). In *Drosophila* larvae, there are 21 OSNs that express ~25 OR genes, of which only 13 are also expressed in adults (Kreher et al. 2005; Ramaekers et al. 2005), suggesting that the olfactory space of the larva is substantially different from the adult one.

# **ABBREVIATIONS**

ACh:	acetylcholine
AL:	antennal lobe
ARM:	anesthesia-resistant memory
CS:	conditioned stimulus
GPCR:	G-protein-coupled receptor
KC:	Kenyon cell
LN:	local neurons
LP:	lateral protocerebrum
LTM:	long-term memory
MB:	mushroom bodies
MTM:	middle-term memory
NO:	nitric oxide
OBP:	odorant-binding protein
OR:	odorant receptor
OSN:	olfactory sensory neuron
PBP:	pheromone binding protein
SEG:	subesophageal ganglion
STM:	short-term memory

US: unconditioned stimulus

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# 3 Olfactory Information Processing in Moths

S. Shuichi Haupt, Takeshi Sakurai, Shigehiro Namiki, Tomoki Kazawa, and Ryohei Kanzaki

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# 3.1 INTRODUCTION

Insects are the most diverse and abundant animal group, representing more than 70% of all known animal species. They display a range of sophisticated and adaptive behaviors based on the perception of a multitude of stimuli. Within the incoming stream of multimodal sensory information, olfactory signals often serve as key stimuli or releasers for the initiation of behaviors such as orientation toward mating partners, localization of appropriate sites for oviposition, and foraging. The vitally important role of olfaction is a general phenomenon across the animal kingdom.

Insects are valuable model systems in neuroscience due to the balance between the moderate complexity of their nervous systems, a rich behavioral repertoire, and the low cost of maintenance as experimental animals. Insect brains contain on the order of  $10^5$  to  $10^6$  neurons, thus they range slightly above *Aplysia* in this measure, but below *Octopus* (>10<sup>8</sup>), which is comparable to small mammals (mouse: ca.  $5 \times 10^7$ ). For comparison, the human brain contains on the order of  $10^{11}$  neurons. The concept of individually identifiable neurons and small networks as functional units have been vital for understanding insect brains, whose main properties are processing speed, relative simplicity, and elegant design principles.

Moreover, insects are well suited for multidisciplinary studies in brain research involving a combined approach at various levels, from molecules to single neurons to neural networks, behavior, and modeling. These preparations are amenable to a wide variety of methodological approaches, in particular genetic engineering, neuroanatomy, electrophysiology, and functional imaging. The similarity in the construction principles of central olfactory processing areas between insects and vertebrates and the common structural units of olfactory processing, called glomeruli, have made insects valuable model systems for investigating general mechanisms of olfactory information processing (Hildebrand and Shepherd 1997; Rössler et al. 2002). The striking similarity in the design of olfactory systems suggests that there are optimized solutions to deal with this kind of stimulus space, whose relevant metrics are still poorly understood. Odor-induced behaviors and their plasticity in insects have also led to important advances in the understanding of learning and memory (Menzel 2001). Even on shorter timescales of odor-induced orientation, flexibility and reliability are features that characterize insect behavior. In particular, moths have been a model system with a long-standing tradition being able to localize a female or pheromone source over long distances in natural environments despite (or because of?) the intermittent stimulus characteristics caused by turbulent flows.

In the present context, we cover, without claiming an exhaustive review of the vast literature, the current state of knowledge concerning moth olfactory behaviors, their plasticity, and the underlying neural mechanisms. These encompass the structure and function of olfactory sensory organs, the molecular mechanisms of olfactory transduction, and the anatomical and physiological properties of olfactory neurons and circuits in the brain, which deliver outputs for the control of behavior.

While there is also a large body of work on the development of the olfactory system in moths that is important for our understanding of the generation of the structural characteristics of olfactory systems, we refer the reader to available reviews covering this topic (Keil 1992; Oland and Tolbert 1996; Salecker and Malun 1999; Tolbert et al. 2004).

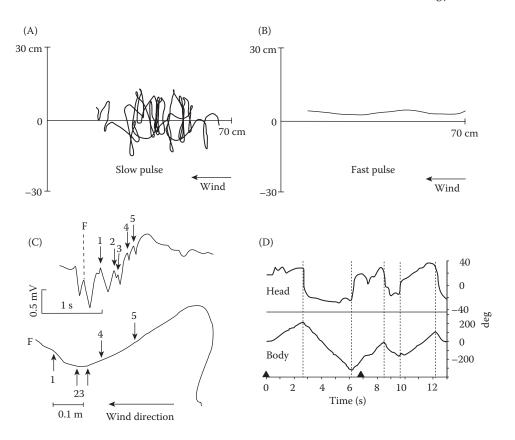
#### 3.2 OLFACTORY BEHAVIOR

Insects are well known for their rich repertoire of olfactory behaviors (Hartlieb and Anderson 1999). Odorants important for moth behavior may be classified as either species-specific pheromones or so-called general odors that are not involved in intraspecific communication. In broad terms, olfactory-induced behaviors in moths can be associated with the contexts of reproduction or foraging and feeding. While olfaction is the dominant modality in reproductive behaviors, visual cues can take precedence in foraging when approaching flowers (Balkenius et al. 2006; Goyret et al. 2007). A factor that must always be considered is that under natural conditions, olfactory stimuli have peculiar properties: they are discontinuous in time at a fixed spatial location, in the form of odor plumes (Murlis et al. 1992).

## **3.2.1 PHEROMONE-RELATED BEHAVIORS**

Sex pheromones released by female moths represent a special class of odors because their signals are processed by conspicuous sexually dimorphic structures in the male moth nervous system (see Section 3.5.1.1). Males are attracted to substances produced by females. In some cases, as in *Bombyx mori* (Butenandt et al. 1959; Kramer 1975), a single component from the mixture released can be behaviorally effective, while in others, such as *Manduca sexta* (Tumlinson et al. 1989), a particular mixture of components, a blend, is necessary (Roelofs 1995). Besides sex pheromones, oviposition pheromones have been found in larval feces, acting as deterrents (Anderson et al. 1993).

Male moths use specific strategies for localizing pheromone sources. Flying moths display a characteristic orientation behavior toward pheromone sources that is supported by optomotor anemotaxis (Kennedy and Marsh 1974), a topic that has been a major focus of research in moth olfactory studies (Baker 1986; Arbas 1997; Baker and Vickers 1997; Cardé and Mafra-Neto 1997; Willis and Arbas 1997; Witzgall 1997). Upon exposure to pheromones, moths take flight upwind in a zigzagging pattern of straight segments at small angles with respect to the wind direction (so-called surge) and counterturns that change into crossing the wind at large angles with little or no net progression (socalled casting) upon odor loss (Kennedy 1983; Figure 3.1A through C). The properties of the behavior are species-specific and also depend on pheromone (blend) concentration (Cardé and Hagaman 1979; Kuenen and Baker 1982; Willis and Arbas 1991; Mafra-Neto and Cardé 1995; Justus and Cardé 2002). Some evidence, however, has led to the postulation of an intrinsic wind-independent turn-generating mechanism (Kennedy et al. 1980; Baker and Kuenen 1982; Kuenen and Baker 1983; Baker et al. 1984). Pheromone orientation should be evaluated in the context of realistic stimulus conditions (Vickers 2000, 2006; Cardé and Willis 2008) and indeed, efficient pheromone source localization depends on temporally fluctuating pheromone concentrations encountered by a moth (Kennedy et al. 1980; Willis and Baker 1984; Baker et al. 1985), with counterturn rates decreasing with increasing pulse frequency, inducing straighter upwind flight paths (Mafra-Neto and Cardé 1994; Vickers and Baker 1994; Figure 3.1A through C). These results support the idea that the flight pattern may consist of two basic elements, surges and casts, internally generated following the first interception of the pheromone stimulus. It should be noted that intermittency of the stimulation also appears to be necessary for the suppression of casting. The combination of surges and casts, the involvement of optomotor anemotaxis, as well as the temporal pheromone stimulation pattern, is thought to result in the behaviors observed. Essentially, this view corresponds to Baker's (1990) model in which surges are initiated by the encounter with strands of pheromone, and casting is initiated upon the encounter of pockets of clean air of sufficient duration in the plume (see also Figure 3.1C). Kaissling and Kramer (1990) also put forward similar concept. However, some moths show regular turning spontaneously without an olfactory stimulus, and their behavior suggests that odor source localization is an actively generated and continuously modulated ongoing pattern of upwind surges and counterturns matched for sampling sensory information (Willis and Arbas 1997, 1998). As a result, the detailed mechanisms of the generation of pheromone orientation behavior are still a matter of debate (Vickers 2006).



**FIGURE 3.1** Orientation of male moths to pheromone stimulation. (A) *Cadra cautella* male orienting in a wind tunnel in response to a pheromone plume pulsed at low frequency. (B) as (A) but with higher pheromone stimulus pulse frequency. (C) Flight track of a *Heliothis virescens* male (bottom) and electroantennogram (EAG) recording (showing the response of olfactory sensory neurons) from an isolated male antenna attached to the flying moth to monitor pheromone filament interception. EAG response onsets are labeled by numbers corresponding to positions in the flight track. The moth takes flight (F) upon the onset of the second EAG response, performs a surge oriented almost in the upwind direction, and upon prolonged loss of the pheromone filament, starts a counterturn. (D) Head turn angle and body orientation in male *Bombyx mori* walking in response to pheromone stimulation (arrowheads). It can be seen that the animal performs counterturns that are correlated with head turns. ([A] and [B] Redrawn from Mafra-Neto, A. and Cardé, R. T., *Nature*, 369, 142–44, 1994. With permission. [C] Redrawn from Vickers, N. J. and Baker, T. C., *Proc. Natl. Acad. Sci.* USA, 91, 5756–60, 1994. With permission. © 1994, National Academy of Sciences, USA. [D] Redrawn after Mishima, T. and Kanzaki, R., *J. Comp. Physiol.* A, 183, 273–82, 1998. With permission.)

After a male moth has located a female, various cues, including male pheromones, are involved in courtship prior to copulation (Krasnoff et al. 1987; Charlton and Cardé 1989).

The flightless male *B. mori* has been particularly useful to investigate pheromone orientation, as there are hardly any other behaviors that the males engage in and movements are restricted to two dimensions in this species (Kanzaki 1997). Despite the similarities with flying moths and the proposal of a unified model (Kramer 1997), the mechanisms are not necessarily identical: for instance, in response to pheromones, flying *Grapholita molesta* show counterturning while they orient in rather straight upwind paths when walking (Willis and Baker 1987). The locomotor pattern of *B. mori* in pheromone orientation depends on the temporal structure of the stimulation (Kramer 1975, 1986, 1992; Kanzaki et al. 1992). With naturalistic pulsed stimulation, a characteristic zig-zag upwind walking pattern is observed (Figure 3.1D), which can also be supported by wing-beat induced displacement after leg ablation (Kanzaki 1998). As in flying moths, there is evidence for

an internal turn generator (Kanzaki et al. 1992). The elementary sequence of pheromone-induced programmed behavior in response to a single pulse is thought to consist of a short straight surge, followed by turning and counterturning, and finally by cycloids (looping). Over some frequency range, zigzag walking is induced, but paths become straighter with increasing frequency. The locomotor pattern is associated with abdominal bending, neck turning, antennal posture, and wing posture responses (Olberg 1983; Kanzaki 1998; Mishima and Kanzaki 1998; Figure 3.1D).

## 3.2.2 BEHAVIORAL RESPONSES TO GENERAL ODORS

The involvement of general odors (i.e., nonpheromonal odorants) in moth behavior has been studied to a lesser extent than pheromone-induced behavior. Among general odors, host-plant-related odorants must be given a special status as they are directly related to reproductive behavior. The interactions between insects and host plants are highly complex and include, for instance, the use of host-plant products as pheromone precursors, the induction of pheromone production by host-plant odors, and the modulation of pheromone responses (Reddy and Guerrero 2004).

Female moths are generally attracted by compounds released by the host plants in order to lay eggs on them (Honda 1995; Natale et al. 2004; Tasin et al. 2005). The orientation behavior of females toward host-plant and other odors has properties largely similar to the orientation of males toward female pheromones in *M. sexta* (Willis and Arbas 1991; Mechaber et al. 2002), implying the same underlying mechanism that is likely to be generally implemented, whether the odorant is a pheromone or any other attractive volatile compound.

In addition to orientation toward host plants, oviposition is also stimulated by host-plant odors (Tichenor and Seigler 1980; Mechaber et al. 2002), and different compounds can be responsible for the upwind flight response and the induction of oviposition. However, tarsal contact, i.e., taste, appears to be the dominant modality in eliciting egg laying (Renwick and Chew 1994; Honda 1995).

Besides host-plant odors, floral odors are important for foraging moths (Haynes et al. 1991; Heath and Manukian 1992). Moths can have innate preferences for odors of flowers (Cunningham et al. 2004), especially those for which they are important pollinators, but interestingly, moths appear to be generally attracted by natural floral cues and by odors in particular (Riffell et al. 2008). Recently, it was also shown that moths are attracted to elevated  $CO_2$  levels, such as those emitted from some newly opened flowers that are likely to contain larger amounts of nectar (Thom et al. 2004). Similarly, water vapor has attractive properties (Raguso et al. 2005), although this is most likely not an olfactory stimulus as far as the sensory apparatus is concerned, the actual mechanism of hygroreception still being a matter of debate (Tichy and Loftus 1996).

## 3.2.3 MODULATION AND PLASTICITY OF OLFACTORY BEHAVIORS

Responses to odors are by no means static. Variations in behavioral sensitivity can occur under various circumstances. One aspect of sensitivity variations is the interaction of different odors or of odors and other sensory modalities. Additionally, endogenous rhythms affect olfactory responsiveness. Nonassociative plasticity can occur due to repeated or lasting exposure to an odor or depending on behavioral or developmental state. Associative learning processes are another factor that can alter behavioral responses to odorants.

## 3.2.3.1 Interaction of Sensory Stimuli

Under natural conditions, a mixture of many odorants is likely to represent realistic stimulus conditions. For instance, the presence of heterospecific pheromones can interfere with pheromoneorientation behavior (Baker et al. 1998). This kind of olfactory interference can specifically affect particular components of behavior (Coracini et al. 2003). On the other hand, synergistic effects have also been reported for some host-plant-related compounds in pheromone-induced behavior (Landolt et al. 1994; Yang et al. 2004; Namiki et al. 2008, see Section 3.3.3.1). Interactions can occur with other stimulus modalities. For instance, the combination of visual and olfactory stimuli can result in increased host-finding behavior compared to the presentation of either stimulus alone (Rojas and Wyatt 1999). In *M. sexta*, either floral odor or the visual aspect of flowers can attract moths toward flowers, but the association of visual and olfactory stimuli is required for actually visiting the flowers (Raguso and Willis 2002, 2005). When exposed to pheromones, olfaction may take precedence over other stimuli. Notably, the auditory-evoked avoidance of bat sounds has been demonstrated to be decreased during pheromone exposure (Skals et al. 2005). However, even a basic behavior such as pheromone orientation relies on multimodal interactions, using optomotor anemotaxis during flight, which requires functional Johnston organs—antennal mechanoreceptors that can detect Coriolis forces—for stabilization (Sane et al. 2007).

## 3.2.3.2 Biogenic Amines and Photoperiod

Injections of biogenic amines, in particular serotonin and octopamine, into the hemolymph of various moth species have sensitizing effects on the male pheromone response and random activity (Linn 1997; Gatellier et al. 2004; see Section 3.3.3.2). In some species, the effectiveness of these compounds depends on the relative timing of the application and the photoperiod (Linn 1997). Generally, the sensitivity to pheromone also varies with the photoperiod (Shorey and Gaston 1965; Cardé et al. 1975; Lindgren et al. 1977; Sasaki and Riddiford 1984; Linn 1997; Rosén et al. 2003; Silvegren et al. 2005), showing a positive correlation with brain serotonin levels in some moths (Kloppenburg et al. 1999; Gatellier et al. 2004), but a negative correlation in others (Linn 1997). Circadian rhythmicity in biogenic amine levels is a possible mechanism at the base of the observed changes of sensitivity. Interestingly, the circadian rhythmicity, in the absence of other external Zeitgeber, can be modified by periodic exposure to pheromone (Silvegren et al. 2005).

## 3.2.3.3 State-Dependent and Nonassociative Plasticity

Variations in the sensitivity of the behavioral response to pheromones following brief pre-exposure are a form of nonassociative plasticity. In a number of moth species, decreases of behavioral sensitivity lasting at least several hours after pre-exposure to high pheromone concentrations have been reported (Traynier 1970; Bartell and Roelofs 1973; Judd et al. 2005). In Trichoplusia ni, a pulsed pre-exposure regime resulted in reduced behavioral responses as compared to continuous pre-exposure. It has been suggested that this habituation occurs at the central nervous system level (Kuenen and Baker 1981). In contrast, behavioral sensitivity was shown to be increased by brief pre-exposure for about one day in Spodoptera littoralis, which was more effective when using the pheromone blend rather than its main component alone (Andersson et al. 2003, 2007). Following mating, the sensitivity to pheromones is transiently decreased (Gadenne et al. 2001). Such statedependent nonassociative plasticity can also be seen in female moths. Depending on the species, virgin females may not be at all attracted to host-plant odors or may be attracted at a lower rate than mated or even aged virgin females (Phelan and Baker 1987; Rojas 1999; Mechaber et al. 2002; Masante-Roca et al. 2007). The response to host-plant odors also shows seasonal variability (Piñero and Dorn 2007). A preference for non-host-plant odors after pre-exposure was induced in females (Zhang et al. 2007), which could also gradually accept or even prefer to oviposit in the presence of initially aversive non-host-plant odors (Liu et al. 2005; Wang et al. 2008). A similar type of change could even be detected across developmental stages and generations: larval exposure to oviposition deterrents, including an odor, increased the acceptance of host plants treated with these deterrents by the imagines and even improved the growth rate of the resulting larvae, as compared to those born from initially unexposed individuals (Akhtar and Isman 2003). A form of cross-modal sensitization has also been reported. In M. sexta, pre-exposure with an attractive olfactory stimulus as a cue for foraging enhances the attractiveness of a subsequently presented visual target (Goyret et al. 2007).

#### 3.2.3.4 Associative Plasticity

Proboscis extension response (PER) conditioning, using sucrose as unconditioned stimulus, is a commonly used learning paradigm in the honeybee (Menzel 2001). The essential features of classical conditioning in moths are similar to those in honeybees. Moths have been conditioned to plant odors and have been shown to be able to discriminate them in differential conditioning (Hartlieb 1996; Fan et al. 1997; Fan and Hansson 2001; Skiri et al. 2005a). Surprisingly, the PER in both sexes could also be conditioned to female sex pheromones, with females performing better (Hartlieb et al. 1999, in *S. littoralis*). In this paradigm, bitter-tasting substances are potential negative reinforcers (Jørgensen et al. 2007). An alternative feeding response, the cibarial pump response, which serves to suck in liquids through the proboscis, could be conditioned in the same way as the PER, but may offer advantages as it is always evaluated by electromyography (Daly and Smith 2000; Daly et al. 2001b, 2008). Generalization can occur when odors used for conditioning have similar molecular properties (Daly et al. 2001a).

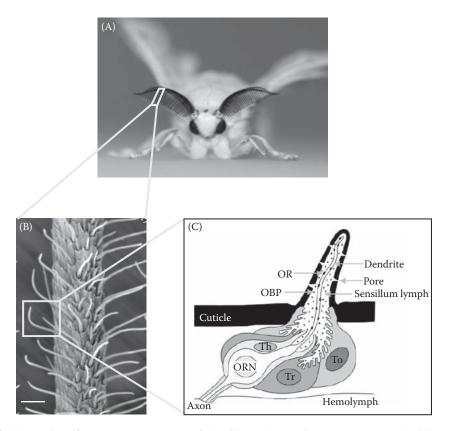
Associative learning closer to behavior in the field has also been employed using the attraction of flying moths to odor sources in which learned odors could take precedence over innately preferred odors. Under such conditions, discrimination of odor mixtures that only differ in one component was possible (Cunningham et al. 2004). Under field conditions, these learning performances are probably reflected by the fact that moths can learn to exploit nectar sources according to their availability, while at least partially retaining innate preferences (Riffell et al. 2008). However, in the case of  $CO_2$ , the innate preference could be reversed by negative experience (Thom et al. 2004). Conditioning experiments with various odorants have recently shown that odor detection and discrimination thresholds are optimized by  $\gamma$ -aminobutyric acid (GABA)ergic inhibition (Mwileria et al. 2008).

# 3.3 PERIPHERAL OLFACTORY PROCESSING

At the base of mechanisms generating olfactory behavior are the neural substrates detecting and processing olfactory information. This sequence of events starts with the reception of odorant molecules by sensory organs. The insect cuticle is covered by specializations of numerous morphological types for sensing chemical, thermal, and mechanical stimuli, sensory organs known as sensilla (Altner and Prillinger 1980). Among the body appendages, antennae assume a special role as compound multimodal sensory organs with a large number of olfactory sensilla. In moths, the most common type of olfactory sensillum has the shape of a hair characterized by the presence of numerous minute pores on its surface. Each sensillar lumen. Odorant signals detected at the dendritic membrane are transduced into electric signals and transmitted to the brain. In this section, we summarize olfactory processing occurring at the peripheral level with an emphasis on the recent progress in the understanding of the molecular mechanism of pheromone reception.

# 3.3.1 STRUCTURE OF OLFACTORY SENSILLA

Insect ORNs are bipolar and housed in cuticular specializations characterized by multiple pores (Figure 3.2) (reviewed in Keil 1999; Steinbrecht 1999). The size of the pores is in the range of 10–100 nm, which is suited to allow the passage of odorants while preventing dessication of the sensillum. The number of ORNs in a single sensillum is two to four in many cases. Three types of auxiliary cells surround the cell bodies and inner dendrites of ORNs: the tormogen, trichogen, and thecogen cells (Figure 3.2C). Transport processes in the auxiliary cells and septate junctions between them and tight contacts of the tormogen cell with the adjacent cuticle give rise to a lymph space surrounding the outer dendrites of the ORNs that is isolated from the hemolymph. Differences in chemical composition of the sensillum lymph and the hemolymph bring about a standing electrical potential difference, the transepithelial potential (TEP) (see Morita and Shiraishi 1985; Kijima et al. 1995). Odorant stimulation generates a receptor potential in the outer dendritic membrane, which



**FIGURE 3.2** Main olfactory sensory organs of the silk moth, *Bombyx mori*. (A) A male silk moth with its prominent antennae optimized for odorant detection. (B) Scanning electron micrograph of the antenna, displaying the external morphology of sensilla trichodea. Scale bar:  $25 \,\mu$ m. (C) Schematic of an olfactory sensillum showing the detailed configuration of ORNs and auxiliary cells with respect to the cuticular specializations. The cell bodies of ORNs are surrounded by three types of auxiliary cells, the tormogen (To), trichogen (Tr), and thecogen cells (Th), which secrete odorant-binding proteins into the sensillum lymph. Odorants are detected by OR expressed on the dendritic membrane of ORNs. ([C] Modified from Jacquin-Joly, E. and Merlin, C., *J. Chem. Ecol.*, 30, 2359–97, 2004, and Steinbrecht, R. A., Ozaki, M., and Ziegelberger, G. *Cell Tissue Res.*, 270, 287–302, 1992.)

can induce the generation of action potentials in a more proximally located spike-generating zone. Olfactory sensilla are categorized into several types by their outer shape (*s. trichodea, s. basiconica, s. coeloconica, s. placodea,* etc.), while the basic structure is conserved among them.

# 3.3.2 Physiology of Olfactory Receptor Neurons (ORNs)

In moths, two types of olfactory systems have evolved: a pheromone-sensing system tuned to species-specific pheromones, and a general odorant-sensing system tuned to nonpheromonal odorants such as host-plant or food-derived odorants.

Pheromones are detected by ORNs housed in long *s. trichodea* on the male moth antennae (Kaissling 1987). For example, the long *s. trichodeum* on the male antennae of the silk moth, *B. mori*, has a pair of ORNs, one being activated by bombykol and the other by bombykal, the two sex pheromone blend components of this species (Kaissling et al. 1978). Such pheromone receptor neurons show extremely high selectivity and generally only respond to a single component of a pheromone blend. In addition, long *s. trichodea* can have ORNs specifically responding to pheromone

components of other, sympatric species to form the physiological basis of behavioral antagonism in males, ensuring reproductive isolation (Mustaparta 1995). These ORNs are cocompartmentalized in long *s. trichodea* in a stereotyped combination (Hansson 1995). Cocompartmentalization of ORNs that are tuned to each of such compounds is required to optimize spatiotemporal resolution (Todd and Baker 1999). Consistent with a functional role to detect pheromones and interspecific signals, in most moth species the long *s. trichodeum* is present only on the male antennae. In several moth species, electrophysiological responses to their own pheromone compounds can be recorded in females (den Otter et al. 1978; Hansson et al. 1989; Ochieng et al. 1995; Seabrook et al. 1987). For instance, part of the short *s. trichodea* on the antennae of female *Heliothis virescens* has ORNs narrowly tuned to their own pheromones (Hillier et al. 2006). Response spectra of ORNs to host-plant odorants suggest that there are two categories of ORNs for general odorants: ORNs narrowly tuned to single or structurally related compounds and ORNs broadly tuned to various compounds (Rostelien et al. 2000a, 2000b; Shields and Hildebrand 2001; Hiller et al. 2006).

In response to odorant stimuli, ORNs generally exhibit phasic-tonic firing patterns. Temporal response patterns of ORNs vary according to odorant concentration and, in some cases, the same odorant may have diverse effects depending on the ORN type stimulated (Shields and Hildebrand 2001). Two response modes exist, excitatory and inhibitory, which are thought to result from depolarizing and hyperpolarizing ionic currents, respectively (de Bruyne et al. 1999). Such response properties are likely to improve resolution in olfactory information encoding and to enhance odorant discrimination.

# 3.3.3 MODULATION OF OLFACTORY RECEPTOR NEURON (ORN) ACTIVITY

# 3.3.3.1 Host-Plant Odors

The physiological activity of ORNs can be influenced by both internal and external factors. As an external factor, host-plant odors are known to modulate the ORN activity of male moths. In *Helicoverpa zea*, ORN responses to the major pheromone component were enhanced by costimulation with an otherwise neutral host-plant odorant (Ochieng et al. 2002). Such enhancement of ORN responses can at least partly explain increased behavioral sensitivity to mixtures of pheromones and host-plant odors (see Section 3.2.3), because increased behavioral sensitivity to stimulation with pheromone in a mixture with a normally neutral host-related compound depended on the application to the same area of the antenna in male *B. mori* (Namiki et al. 2008). In either case, the host-plant odorants alone did not induce behavioral or physiological responses. Of particular interest is examining the molecular target of such synergistic effects of pheromone and plant odorant in ORNs.

# 3.3.3.2 Circadian Rhythms

In accordance with the diel rhythm of female calling behavior (e.g., Rosén 2002), male moths display sensitivity variations in their pheromone responsiveness (see Section 3.2.3.2) that have been suggested to be due to modulation at central rather than peripheral targets (Rosén et al. 2003). However, recent studies revealed diel changes in the antennal sensory responsiveness to pheromones in *M. sexta* (Flecke et al. 2006) and *S. littoralis* (Merlin et al. 2007). In support of a physiological rhythmicity, the expression of a clock protein has been confirmed in ORNs of *M. sexta* (Schuckel et al. 2007). Furthermore, the expression level of transcripts of the clock genes *period, cryptochrome1* and *cryptochrome2* showed circadian rhythms in the antennae as well as in the brain of *S. littoralis* (Merlin et al. 2007), suggesting that the circadian oscillation of clock gene expression in ORNs is responsible for circadian rhythms in ORN sensitivity.

# 3.3.3.3 Biogenic Amines

A class of internal agents known to modulate ORN activity is biogenic amines. In three moth species, exogenous octopamine enhanced spike responses of pheromone-specific ORNs (Pophof 2000,

2002; Grosmaitre et al. 2001). Only in *B. mori* an increase in receptor potential amplitude could also be observed (Pophof 2002). The expression of an octopamine receptor in the cells adjacent to olfactory sensilla has been confirmed by *in situ* hybridization analyses (Von Nickisch-Rosenegk et al. 1996). The subcellular localization of octopamine receptors is now necessary to identify the target(s) of octopamine involved in the modulation of ORN sensitivity. Another biogenic amine, serotonin, has been reported to induce effects opposite to those of octopamine (Grosmaitre et al. 2001). In *M. sexta*, octopamine and serotonin also influenced the TEP, a standing potential difference between sensillar lymph and hemolymph space in insect sensilla, showing that the accessory cells that generate this potential difference are targets of aminergic modulation (Dolzer et al. 2001). A direct link between peripheral modulation by biogenic amines and circadian and other types of sensitivity modulation has not been established so far, although exogenous octopamine has clear behavioral effects (Linn and Roelofs 1986; Linn et al. 1992, 1996; Linn 1997, see Section 3.2.3.2).

# 3.3.4 MOLECULAR MECHANISM OF ODORANT RECEPTION

Moth antennae, especially in males, are often carefully tuned systems to optimize odorant catch (Adam and Delbrück 1968; Kaissling and Priesner 1970; Koehl 2006). Once odorants are absorbed on the cuticular surface, they can diffuse inside the sensilla through sensillar pores. When odorants enter the sensillum lymph surrounding the dendritic membrane of ORNs, two kinds of processes occur. At first, perireceptor events (Getchell et al. 1984) take place in proximity of the ORNs and determine the residence time of odorants in the sensillum lymph, as well as the efficiency of odorant transfer to the ORN membrane. After these processes, receptor events occur by the specific interaction of odorants with olfactory receptor proteins (ORs), which lead to the activation of the chemoelectric transduction machinery in the ORN. Although recent evidence has accumulated pointing toward the specific interaction of odorants with ORs as the critical step for the detection and discrimination of odors (de Bruyne and Baker 2008), the understanding of perireceptor events is also vital.

# 3.3.4.1 Perireceptor Events

## 3.3.4.1.1 Odorant-Binding Proteins

Due to the hydrophobic nature of volatile odorants, the aqueous sensillum lymph represents a hydrophilic barrier impeding the diffusion of odorants toward the dendritic membrane of ORNs. This problem has been resolved by the expression of small (about 15 kDa) soluble proteins, termed odorant-binding proteins (OBP), which are extremely abundant in the sensillum lymph and thought to bind and transfer odorants to the ORNs (Pelosi et al. 2006). The first OBP described was discovered in the sensillum lymph of Antheraea polyphemus (Vogt and Riddiford 1981). OBPs have since been found in many insects, including numerous moth species (Pelosi et al. 2006). In moths, OBPs are grouped into four classes based on their amino acid sequence similarity: pheromone-binding proteins (PBPs), two types of general odorant-binding proteins (GOBP1 and GOBP2), and the antennal binding protein X (ABPX; Vogt et al. 1991, 1999). These proteins are synthesized by tormogen and trichogen cells, which secrete them into the sensillum lymph (Laue and Steinbrecht 1997). Their expression pattern and close relationship with particular sensillum types indicate a functional differentiation of the OBPs. In general, PBPs are predominantly expressed in the male antennae and are localized in the sensillum lymph of pheromone-sensitive s. trichodea, while other OBPs are expressed at a similar level in both the male and female antennae in s. basiconica (Steinbrecht et al. 1995), which are believed to respond to plant-derived odorants.

OBPs are thought to function as passive carriers for odorants. For instance, PBP undergoes a conformational change when the pH becomes more acidic, as would be expected to occur by virtue of the fixed negative charges on the cell membrane. This conformational change might result in the release of the bound pheromone onto the dendritic membrane of the ORN (Wojtasek and Leal 1999). This hypothesis has been supported by subsequent structural analyses showing that

conformational changes at acidic pH lead to the release of bound bombykol in *B. mori* PBP (Tegoni et al. 2004). Besides their function as odorant carriers, OBPs have been suggested to be involved in odorant discrimination by functioning as a molecular filter for odorants to cross the sensillum lymph, and may also have a role in the activation of ORs (Kaissling 2001; Pophof 2004). While the specificity of ORs could be enhanced by OBPs (Große-Wilde et al. 2006), the latter are not essential for the activation of ORs and specific responses of ORNs, since heterologous expression of ORs in the absence of OBPs results in responses to odorants as selective as in in vivo ORNs (Wetzel et al. 2001; Sakurai et al. 2004; Nakagawa et al. 2005; Große-Wilde et al. 2006).

# 3.3.4.1.2 Odorant Degrading Enzymes

For efficient orientation of male moths toward females, the capability of following an intermittent pheromone trail comprising pockets of pheromone-free air is paramount. This requires sufficiently high temporal resolution of the male sensory apparatus. In fact, electrophysiological recordings under pulsed pheromone stimulation revealed that the temporal resolution of male antennae is in the range 5-33 Hz (Rumbo and Kaissling 1989; Marion-Poll and Tobin 1992; Bau et al. 2002, 2005). Thus, after activating ORs, odorants must be inactivated and eliminated rapidly to maintain high sensitivity to incoming stimuli. To accomplish such a rapid inactivation of odorants, the sensillum lymph contains odorant degrading enzymes (ODE) that enzymatically modify odorants into inactive substances. Two types of ODEs for pheromones (PDE: pheromone degrading enzyme) have been characterized in sensillum lymph; a sensillar esterase in A. polyphemus (Vogt et al. 1981, 1985) and an aldehyde oxidase in *M. sexta* (Rybczynski et al. 1989). Based on the kinetics of these enzymes, the half-life of pheromone molecules in sensillum lymph was estimated to be 15 and 0.6 ms in A. polyphemus and M. sexta, respectively (Vogt et al. 1985; Rybczynski et al. 1989). Recently, a gene encoding a sensillar esterase with properties similar to that previously isolated was identified from A. polyphemus male antennae, and named ApolPDE (Ishida and Leal 2005). The enzymatic efficiency of purified ApolPDE is about fortyfold higher than that of partially purified PDE by Vogt et al. (1985). The properties of ApolPDE are sufficient to explain the temporal resolution observed in physiology and behavior. Furthermore, the authors showed that the kinetics of pheromone degradation by ApolPDE were slowed at acidic pH, which may prevent degrading pheromones released from PBPs in close proximity to the dendritic membrane (Ishida and Leal 2005). In contrast to PDE, little research has been concerned with ODEs for general odorants, which have, so far, not been reported in moths.

# 3.3.4.2 Olfactory Receptors (ORs) in Moths

Odorants delivered by OBPs are bound by ORs in the dendritic membrane of ORNs. ORs in insects were first identified in Drosophila melanogaster through genome surveys of 7-transmembrane receptors (Clyne et al. 1999; Vosshall et al. 1999). In moths, ORs have been identified in several species (Krieger et al. 2002, 2004; Sakurai et al. 2004; Nakagawa et al. 2005; Wanner et al. 2007; Mitsuno et al. 2008). Although insect ORs are predicted to be 7-transmembrane proteins like vertebrate G-protein-coupled ORs (Firestein 2001; see also Chapter 7), there is no relationship in their amino acid sequences with vertebrate ORs or any known G-protein-coupled receptors (GPCRs) (Clyne et al. 1999; Vosshall et al. 1999). The B. mori genome project provided almost the entire genome sequence and 64 candidate OR genes were predicted (Xiang et al. 2009). In D. melanogaster, it has been shown that each ORN expresses a single or a few ORs, and ORNs expressing the same OR(s) convergently project into a single glomerulus to create a topographic map of odor information in the Antennal Lobe (AL) (Vosshall et al. 2000; Gao et al. 2000; Fishilevich and Vosshall 2005; Couto et al. 2005). Such principles may be applicable in the moth olfactory system, as the number of candidate OR genes is well correlated with that of the glomeruli in the AL of B. mori (Kazawa et al. 2009; see also Section 3.5.3). Although the ligands for most of the moth candidate ORs are still unknown, ORs tuned to detect behaviorally relevant odorants, including pheromones and plant odorants, have been described.

In *B. mori*, two male antenna-specific OR genes, *BmOR1* and *BmOR3*, have been identified as sex pheromone receptor genes. *BmOR1* and *BmOR3* are mutually exclusively expressed in pairs of pheromone receptor neurons in long *s. trichodea*, being fine-tuned to bombykol and bombykal, respectively (Sakurai et al. 2004; Nakagawa et al. 2005). These observations are consistent with physiological studies in which one of a pair of pheromone receptor neurons in long *s. trichodea* was activated by bombykol and the other responded to bombykal (Kaissling et al. 1978), and provide evidence that highly selective discrimination of pheromone components is accomplished by ligand selectivity of the ORs.

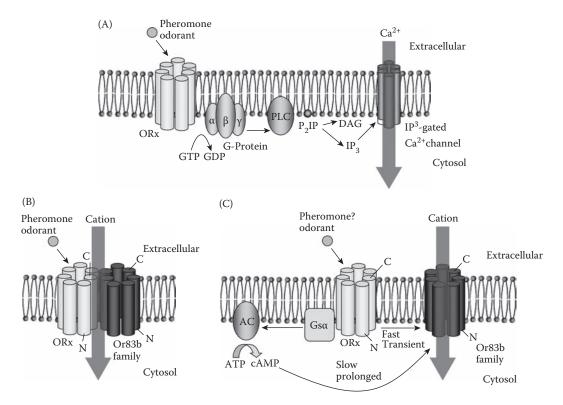
More recently, sex pheromone receptors in *H. virescens* (Große-Wilde et al. 2007), *Plutella xylostella*, *Mythimna separata*, and *Diaphania indica* (Mitsuno et al. 2008) have been functionally identified. Phylogenetic analyses of insect ORs indicate that these genes form a subfamily within the insect OR gene family, suggesting that sex pheromone receptors have evolved from a common ancestral OR gene (Mitsuno et al. 2008).

The function of ORs for general odorants has been less well studied compared to pheromone ORs and, currently, only three ORs (BmOR19, BmOR45, and BmOR47, in *B. mori*) have been characterized to be involved in the detection of plant odorants in moths (Anderson et al. 2009). These ORs are predominantly or exclusively expressed in the female antennae. BmOR19 responds to linalool, which has been reported to elicit characteristic wing fluttering behavior in female moths (Priesner 1979), while the other two ORs respond most strongly to benzoic acid and moderately to several benzyl moiety-containing odorants. BmOR19 expressing ORNs are colocalized with BmOR45 and/ or BmOR47 expressing ORNs within the same sensilla (Anderson et al. 2009). These sensilla are likely to be long *s. trichodea*, because two ORNs in long *s. trichodea* of female silk moths are known to respond to either linalool or benzoic acid (Heinbockel and Kaissling 1996).

#### 3.3.4.3 Signal Transduction Following Odorant Reception

In ORNs, odorant signals are converted into electric activity by a chemoelectric transduction mechanism (Figure 3.3). Transduction was supposed to be mediated by a second messenger cascade triggered by the activation of a heterotrimeric G-protein by ORs with a bound ligand. In this model, odorant-evoked OR activation leads to a conformational change of a heterotrimeric G-protein comprising Gq  $\alpha$  subunits. Thereafter, Gq induces phospholipase C (PLC) activation that results in the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP<sub>2</sub>) into inositol (1,4,5)-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). Subsequent opening of IP<sub>3</sub>-gated Ca<sup>2+</sup> channels induces Ca<sup>2+</sup> influx, which, in turn, opens Ca<sup>2+</sup>-dependent cation channels to generate the receptor potential (Stengl 1994; Krieger and Breer 1999; Jacquin-Joly and Merlin 2004; Jacquin-Joly and Lucas 2005; Figure 3.3A).

Recently, this view was challenged by the finding that insect ORs either form a ligand (odorant)gated nonselective cation channel with an atypical OR, named Or83b family protein (Sato et al. 2008; Figure 3.3B), or that insect ORs directly activate Or83b to function as a nonselective cation channel (Wicher et al. 2008; Figure 3.3C). In these models, the Or83b family protein plays a central role in signal transduction. Or83b was initially isolated from D. melanogaster as a member of the OR gene family (Vosshall et al. 2000), but it has the following two characteristic features that distinguish it from conventional ORs: (1) Or83b is expressed in almost all ORNs, while conventional ORs are expressed in restricted subsets of the ORN population. (2) Or83b family genes are highly conserved among different species including moths (Krieger et al. 2003; Jones et al. 2005), whereas conventional ORs show an extreme diversity in amino acid sequences. In fact, Or83b is not directly involved in odor detection, but supports translocation of coexpressed ORs to the dendritic membranes where it forms a heteromeric complex with ORs (Larsson et al. 2004; Neuhaus et al. 2004). Sato et al. coexpressed BmOR1 with BmOR2, a B. mori Or83b orthologue, and other combinations of members of the Or83b family with ORs in heterologous expression systems. Examination of the electrophysiological properties of an Or83b/OR complex revealed that it acts as an odorant-gated nonselective cation channel (Figure 3.3B) (Sato et al. 2008). Interestingly, there was no evidence for an elevation of second messenger levels upon stimulation with ligands appropriate for the expressed

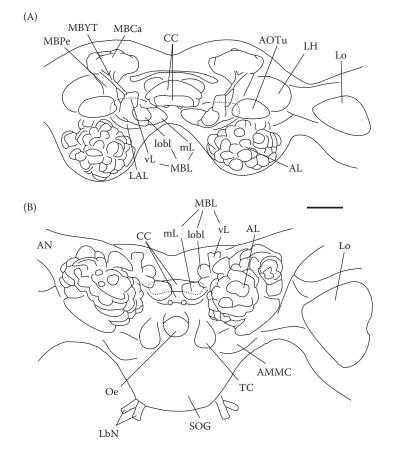


**FIGURE 3.3** Proposed signal transduction cascades following odorant reception. (A) A conventional model of olfactory transduction that involves a G-protein-mediated PLC- $IP_3$  pathway. (B) Alternative model in which the odorant receptor (OR) forms a heteromeric odorant-gated nonselective cation channel with an OR83b family protein. (C) Alternative model postulating two pathways, both depending on a cation channel function of Or83b. An ionotropic pathway involves direct activation of Or83b by an OR with bound ligand inducing a fast transient cation influx. A metabotropic pathway is G-protein-coupled and induces slow, prolonged cation currents. AC: adenylyl cyclase

ORs, implying that there was no involvement of a G-protein-mediated cascade in the activation of Or83b/OR complexes. Wicher et al. (2008) found that fast transient and slow prolonged ionic currents occur in cultured cells coexpressing Or83b and *D. melanogaster* ORs upon stimulation with appropriate ligands. They proposed that the fast currents result from direct activation of Or83b by ORs, and that the slow currents result from G-protein-mediated activation of Or83b (Figure 3.3C). The atypical insect OR family Or83b represents the first identified 7-transmembrane ion channels so far. In this regard, insect ORs have a reversed topology relative to conventional GPCRs with their N-terminus on the cytoplasmic side and the C-terminus on the extracellular side (Benton et al. 2006).

# 3.4 MOTH BRAIN STRUCTURE

After detection by the peripheral processes, olfactory information is relayed to the central nervous system (CNS) to generate behavioral reactions. A short overview of the structure of moth brains is provided here as an introduction (Figure 3.4). Moth brains, as all insect brains, can be divided into four large regions: protocerebrum (PC), deutocerebrum (DC), tritocerebrum (TC), and subesophageal ganglion (SOG), which are fused in moths. The first three form the supraesophageal ganglion. The PC, which includes the optic lobes (OL), belongs to the ocular segment, whereas the DC and TC are associated with the antennal and labral ancestral segments, respectively. The SOG is composed



**FIGURE 3.4** Anatomy of a moth (*Manduca sexta*) brain with emphasis on the olfactory neuropils. (A) View from dorsal (horizontal orientation with respect to body axis). (B) View from frontal (transversal orientation with respect to body axis). AL: antennal lobe; AMMC: antennal mechanosensory and motor center; AN: antennal nerve; AOTu: anterior optic tubercle; CC: central complex; LAL: lateral accessory lobe; LH: lateral horn; LbN: labial nerves; Lo: lobula; lobl: mushroom body lobelet; MBCa: mushroom body calyx; MBL: mushroom body lobes; MBPe: mushroom body pedunculus; MBYT: mushroom body Y tract; mL: mushroom body medial lobe; Oe: esophageal foramen; SOG: subesophageal ganglion; TC: tritocerebrum; vL: mushroom body vertical lobe. Only peripheral nerves known to be relevant for olfaction are shown. The major part of the optic lobes is omitted, except for the lobula. Scale bar: 200 µm. (Modified after Homberg, U., Kingan, T. G., and Hildebrand, J. G. *Cell Tissue Res.*, 248, 1–24, 1987. With permission.)

of three neuromeres representing ancestral segments: mandibular, maxillary, and labial. From the SOG, the neck connective carries ascending and descending information from and to the ventral nerve cord. The overall structure of insect brains and the architecture of some areas important in olfaction have been reviewed in some detail previously (Bullock and Horridge 1965; Strausfeld 1976; Mobbs 1985; Homberg et al. 1989; Hansson and Anton 2000; Fahrbach 2006).

ORNs of the antenna project to the primary olfactory neuropil of the DC, the AL (see Section 3.5). In the AL, a segregation of pheromone and general odor information has been well documented in male moths, which possess a sex-specific macroglomerular complex (MGC) processing pheromone information (see Section 3.5.1). Odor information is relayed to the PC via AL projection neurons (PNs) that project to the mushroom body (MB) and the lateral protocerebrum (LPC), namely to the lateral horn (LH; see Section 3.6). The superior median PC (SMPC) is possibly involved in subsequent olfactory information processing (see Section 3.6.2). The lateral accessory lobe (LAL) represents the major output area of the brain, carrying olfactory signals to more posterior ganglia (see

Section 3.6.2). The antennal system also contains mechanoreceptive and gustatory sensory cells, which project to the antennal mechanosensory and motor center (AMMC) and the SOG (Jørgensen et al. 2006). This deutocerebral area is additionally involved in the motor control of the antenna (Kloppenburg et al. 1997).

The optic ganglia receive visual inputs from the retinae of the compound eyes. This information is relayed through the lamina ganglionaris and the medulla, lobula (Lo), and Lo plate, mostly to the PC, but also to the AMMC as well as to the thoracic ganglia through descending PC neurons. The MBs of moths probably also receive visual information along with other modalities. Further visual and multimodal areas are the anterior optic tubercle (AOTu) and the central complex (CC). So far, these PC areas and the ocellar pathway have not been studied in great detail in moths. The TC and SOG have also received little attention in moths so far. The SOG contains at least circuitry related to the sensory and motor function of the mouth parts and the neck (Kvello et al. 2006; Mishima and Kanzaki 1998). There are olfactory projections from the labial pit organ, bearing  $CO_2$ -sensitive sensilla to the AL (Bogner et al. 1986; Kent et al. 1986). Whether projections in the TC and the SOG seen in these studies are olfactory remains to be investigated.

In moths, as in other insects, the major excitatory neurotransmitter in the CNS is acetylcholine, which is also the candidate neurotransmitter of ORNs (Homberg and Müller 1999). Glutamate is thought to function as a CNS neurotransmitter (Sinakevitch et al. 2008). In the brain, inhibition is conveyed by GABAergic neurons (Homberg et al. 1987; Iwano and Kanzaki 2005; Seki and Kanzaki 2008). Nitric oxide is also implicated in signaling (Nighorn et al. 1998; Seki et al. 2005). Furthermore, the biogenic amines, octopamine, tyramine, dopamine, serotonin, and histamine, as well as neuropeptides have been detected in moth brains (Homberg et al. 1987, 1990, 1991; Homberg and Hildebrand 1989, 1991; Iwano and Kanzaki 2005; Dacks et al. 2005; Sjöholm et al. 2006; Berg et al. 2007; Sinakevitch et al. 2008).

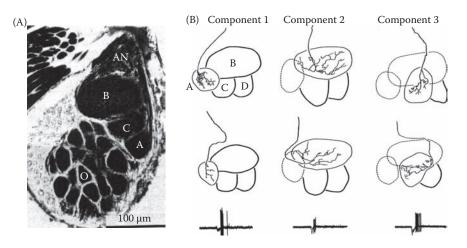
## 3.5 INFORMATION PROCESSING IN THE ANTENNAL LOBE

#### 3.5.1 GLOMERULI AND NEURONAL COMPONENTS IN THE MOTH ANTENNAL LOBE

# 3.5.1.1 Glomerular Organization and Projections of Identified Olfactory Receptor Neurons (ORNs)

Olfactory information from the antennae is conveyed by the central projections of ORN axons to the AL, the main primary olfactory neuropil in the insect brain. The AL is composed of dense compartments of synaptic neuropil, termed glomeruli (Strausfeld 1976; Hildebrand and Shepherd 1997). Detailed morphological maps of the ALs are available for a number of moth species (Rospars and Chambille 1981; Rospars 1983; Rospars and Hildebrand 1992; Berg et al. 2002; Sadek et al. 2002; Greiner et al. 2004; Huetteroth et al. 2005; Masante-Roca et al. 2005; Skiri et al. 2005b; Kazawa et al. 2009). The AL of male moths is divided into two subregions: the MGC, which is an assembly of large glomeruli near the base of the AN, receiving the axons of pheromone-sensitive ORNs (Boeckh and Boeckh 1979; Matsumoto and Hildebrand 1981; Kanzaki and Shibuya 1986; Koontz and Schneider 1987), and the ordinary glomeruli, which are an array of small glomeruli present in both sexes, receiving input from the axons of ORNs tuned to general odors (Hansson 1995). The ordinary glomeruli also comprise glomeruli that process nonolfactory modalities (Guerenstein et al. 2004; Han et al. 2005).

Early imaging studies have revealed that odor stimulation elicits an activity pattern in a specific combination of glomeruli, supporting the concept that the individual glomerulus is the functional unit for olfactory processing (Rodrigues and Buchner 1984). This is in line with the fact that axonal branches of individual ORNs are restricted to single glomeruli. ORNs tuned to particular pheromone components project to particular subdivisions of the MGC, forming a topographic map of pheromone component information in the MGC (Hansson et al. 1992; Hansson 1995; Figure 3.5). The female AL contains two sexually dimorphic glomeruli, the large female glomeruli, in lieu of



**FIGURE 3.5** Axonal projections of functionally identified pheromone receptor neurons in the male turnip moth, *Agrotis segetum*. (A) AL structure of male *A. segetum*. The MGC is situated at the entrance of the AL, and MGC subdivisions are indicated by A, B, and C. A cluster of ordinary glomeruli (O) is visible below the MGC. AN: antennal nerve. (B) Reconstructions of axonal projections of single pheromone receptor neurons. ORNs tuned to different pheromone components terminate in distinct subdivisions of the MGC. Two examples are shown for each ORN type. Electrophysiological responses of the ORNs to cognate pheromone components are shown at the bottom. (From Hansson, B. S., Anton, S., and Christensen, T. A. *Science*, 256, 1313–15, 1992. With permission.)

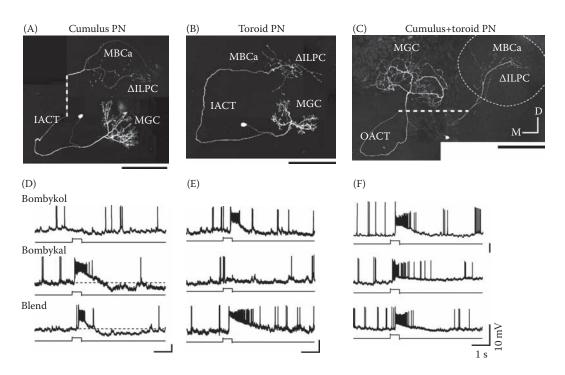
the MGC in *M. sexta* (Rössler et al. 1998) and *H. virescens* (Berg et al. 2002). The large female glomeruli are innervated by ORNs of short s. trichodea that respond to host-plant odors in *M. sexta* (Shields and Hildebrand 2001), but by ORNs tuned to the species-specific pheromone or linalool in *H. virescens* (Hillier et al. 2006).

In addition to the ORN-glomerulus relationship, the innervation pattern of individual pheromone receptor neurons within the MGC can be correlated with the position of the corresponding sensilla on the antennae (Christensen et al. 1995; Ai and Kanzaki 2004). For example, ORNs from the medial and lateral sides of the antennae branched in medial and lateral regions, respectively, in the MGC of *B. mori* (Ai and Kanzaki 2004; see Section 3.5.2.3).

#### 3.5.1.2 Neuron Types in the Antennal Lobe (AL)

Moth ALs contain two major types of neurons besides the axonal projections of ORNs. PNs are principal cells of the AL and transmit olfactory information from the AL to the PC (Figure 3.6). Homberg et al. (1988) have first systematically classified PNs in the AL of *M. sexta*. PNs were classified by morphological characteristics, such as the number of glomeruli that are innervated, the position of the soma, and the tracts in which their axons project (called the antenno-cerebral tracts, ACTs) and later, this classification scheme was applied to other moth species. Uniglomerular PNs have dendritic arborizations restricted to single glomeruli. The majority of the PNs in the moth AL are of the uniglomerular type. Multiglomerular PNs have dendritic ramifications in multiple glomeruli and are less well understood, although several studies have investigated this type of PN in moths (Kanzaki and Shibuya 1986; Homberg et al. 1988; Kanzaki et al. 1989; Rø et al. 2007).

Local interneurons (LNs) are intrinsic cells of the AL and connect individual glomeruli, indicating that LNs have an important role in interglomerular interaction (Figure 3.7). Moth LNs described so far are all spiking neurons, most of them GABAergic, exerting inhibition on both PNs and other LNs identified by anatomical and electrophysiological methods (Waldrop et al. 1987; Christensen et al. 1993, 1998). The AL contains LN populations quite heterogeneous in dendritic morphology and immunohistochemical staining properties (Iwano and Kanzaki 2005; Seki and Kanzaki 2008).



**FIGURE 3.6** Morphology and physiology of MGC-PNs of the silk moth. (A–C) Confocal images of PNs innervating cumulus (A), toroid (B), and both glomeruli (C). The axons of uniglomerular PNs run in the IACT and send blebby projections to the MBCa and the lateral part of the  $\Delta$ ILPC (A, B). The axon of the multiglomerular PN runs in the OACT and sends blebby projections to the  $\Delta$ ILPC and the LH. Scale bar: 100 µm. (D–F) Responses of uniglomerular PNs innervating cumulus (D), toroid (E), and multiglomerular PN innervating both glomeruli (F) to two pheromone components and the blend. The uniglomerular PNs innervating the cumulus or the toroid show selective excitatory responses to bombykal and bombykol, respectively (D, E). The multiglomerular PN responds to both components (F). Dotted lines indicate the resting membrane potential in (D), showing excitatory and inhibitory response periods. D: dorsal; M: medial. (From Kanzaki, R., Soo, K., Seki, Y., and Wada, S. *Chem. Senses*, 28, 113–30, 2003. With permission.)

LNs are classified into several types according to their morphological characteristics (Figure 3.7), but their functional significance in moths remains to be investigated. In addition to PNs and LNs, moth ALs contain several types of extrinsic neurons (Kent et al. 1987; Homberg and Hildebrand 1989; Sun et al. 1993; Hill et al. 2002; Dacks et al. 2005, 2006; see also Section 3.5.5). They normally have several processes covering wide areas of the brain.

# 3.5.2 PHEROMONE PROCESSING IN THE MACROGLOMERULAR COMPLEX (MGC)

# 3.5.2.1 Encoding Pheromone Component Information

Numerous studies have revealed that MGC PNs respond to sex pheromones (Kanzaki and Shibuya 1983, 1986; Christensen et al. 1987; Kanzaki et al. 1989; Anton and Hansson 1994, 1995; Vickers et al. 1998), and that the PNs innervating the same glomerulus in the MGC show similar selectivity for pheromone components (Hansson et al. 1991, 1992; Lei et al. 2002; Kanzaki et al. 2003; Kárpáti et al. 2008; but see Anton and Hansson 1999). Each moth species has a specific glomerular array whose organization is similar, yet distinct even among closely related species (Vickers and Christensen 2003; Vickers et al. 2005). This implies that the response profile of PNs is mainly defined by their input glomeruli. For example, in male silk moth, PNs, which innervate the main MGC glomeruli, named toroid and cumulus, selectively respond to bombykol and bombykal,

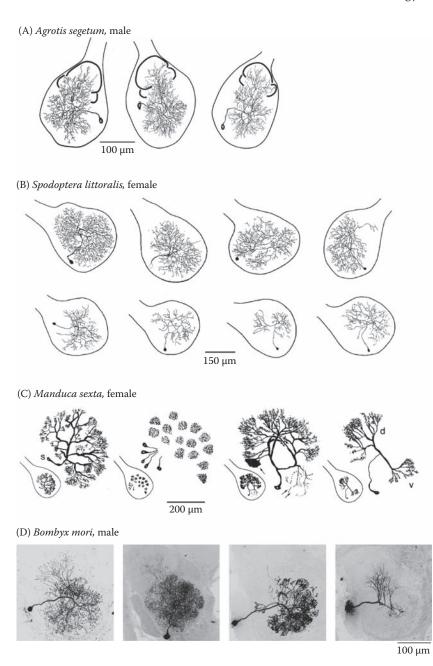


FIGURE 3.7 Various types of LNs in (A) male Agrotis segetum, (B) female Spodoptera littoralis, (C) female Manduca sexta, and (D) male Bombyx mori. ([A] From Hansson, B. S., Anton, S., and Christensen, T. A. J. Comp. Physiol. A, 175, 547–62, 1994. With permission. [B] From Anton, S. and Hansson, B. S., J. Comp. Neurol., 350, 199–214, 1994. With permission. [C] From Christensen, T. A., Waldrop, B. R., and Hildebrand, J. G. J. Comp. Physiol. A, 173, 385–99, 1993. With permission. [D] Modified from Seki, Y. and Kanzaki, R., J. Comp. Neurol., 506, 93–107, 2008. With permission.)

respectively. Thus, selectivity at ORN level is faithfully maintained in the AL, indicating a labeled line-coding scheme.

Multiglomerular PNs have the ability to integrate information about multiple pheromone components. In the silk moth, PNs innervating both main MGC glomeruli show excitatory responses to both pheromone components (Figure 3.6; Kanzaki et al. 2003). Multiglomerular PNs, which only respond to a blend containing all pheromone components, have been found in *Agrotis segetum* and *H. virescens* (Hansson et al. 1994; Christensen et al. 1995; Wu et al. 1996).

# 3.5.2.2 Encoding Temporal Information

Odor stimuli naturally occur in the form of plumes of complex shapes in which odorant concentrations change momentarily (Murlis and Jones 1981; Murlis et al. 2000). The processing of such dynamic information requires that the moth's nervous system operates over a wide frequency band. Intracellular recording studies have shown that PNs can follow pulsed stimuli up to ~10 Hz (Christensen and Hildebrand 1997; Lei and Hansson 1999; Heinbockel et al. 1999, 2004). Even if the temporal pattern of the sensory input is strongly fluctuating, moth PNs can provide a faithful measure of instantaneous pheromone concentration (Vickers et al. 2001). Furthermore, stimulation with blends rather than individual pheromone components increases the capability of PNs to resolve pulsed stimuli (Christensen and Hildebrand 1997; Heinbockel et al. 2004) and affect spike timing in uniglomerular PNs (Lei et al. 2002). PNs innervating different glomeruli in the MGC show synchronized firing in response to pheromone blend. Interglomerular interactions mediated by LNs are likely to be important in temporal processing (see also Chapter 13).

# 3.5.2.3 Encoding Spatial Information

To capture the shape of a plume, discriminating the site of pheromone reception on the antennae seems to be an efficient method. Actually, in the sphinx moth, *M. sexta*, the MGC has a set of PNs that have different spatial selectivity on the antennae, and thus the moth AL has the ability to process somatotopic information at a population level (Heinbockel and Hildebrand 1998). In the silk moth, the somatotopic input organization of the MGC is also reflected at the physiological level (Ai and Kanzaki 2004).

# 3.5.3 GENERAL ODOR PROCESSING IN THE ORDINARY GLOMERULI

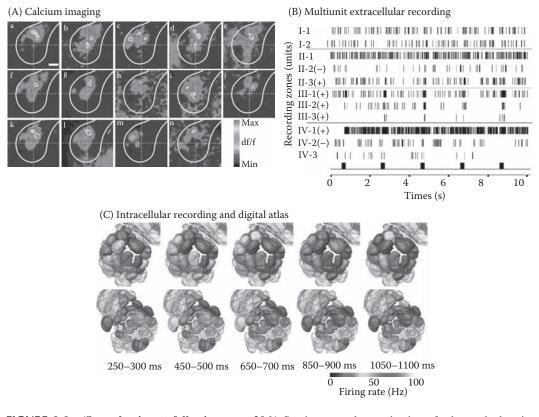
# 3.5.3.1 Combinatorial Olfactory Representation

The ordinary glomeruli are an assembly of ca. 60 glomeruli in the moth AL (Anton and Homberg 1999; Schachtner et al. 2005) and process information from ORNs tuned to general odorants. As in the MGC, PNs innervating the same glomeruli generally have similar olfactory response profiles (Reisenman et al. 2005; Namiki and Kanzaki 2008; *D. melanogaster:* Wilson et al. 2004; Bhandawat et al. 2007; but see Sadek et al. 2002). One major hypothesis is that odor identity is encoded by the combination of active glomeruli.

Odor stimulation evokes spatiotemporal activity patterns in the PN population (Figure 3.8). Optical imaging in vivo has been widely used in the investigation of the moth AL (Okada et al. 1996; Galizia et al. 2000; Carlsson et al. 2002, 2005; Hansson et al. 2003; Meijerink et al. 2003; Skiri et al. 2004; Figure 3.8A). Calcium imaging has revealed a detailed chemotopic representation in the AL (Hansson et al. 2003; Carlsson et al. 2005). While aromatics activated different subregions of the AL as compared to terpenes, compounds in the same class elicited similar activation patterns, a finding that has been confirmed by multiunit recording (Lei et al. 2004; Figure 3.8B). These studies also showed that the spatial representations of odors are dynamic. Using a different approach, the combination of a digital AL atlas and single-cell electrophysiological and anatomical identification, Namiki and Kanzaki (2008) reconstructed the odor-evoked spatiotemporal activity of a PN population in the silk moth (Figure 3.8C). Different odors were shown to elicit distinct spatiotemporal patterns.

# 3.5.3.2 Synchronized Activity

The AL may encode sensory information at multiple timescales. When one monitors the odor-evoked firing rate change in a low frequency band (~1–20 Hz), PNs show various temporal activation patterns (Christensen et al. 2000; Stopfer et al. 2003; Daly et al. 2004; Lei et al. 2004; Namiki and Kanzaki



**FIGURE 3.8** (See color insert following page 206.) Spatiotemporal organization of odor-evoked activity in the moth AL. (A) Olfaction activation pattern in the AL of *Spodoptera littoralis* revealed by calcium imaging. (B) Ensemble olfactory response in the AL of *Manduca sexta* revealed by tetrode recording. (C) Reconstructed geometry of odor-evoked firing activity in the AL of *Bombyx mori*. Anterior and posterior views of reconstructed population activities in response to cis-3-hexen-1-ol. The color of each glomerulus represents the average firing rate from baseline of the innervating PNs. ([A] From Carlsson, M. A., Galizia, C. G., and Hansson, B. S. *Chem. Senses*, 27, 231–44, 2002. With permission. [B] From Lei, H., Christensen, T. A., and Hildebrand, J. G. *J. Neurosci.*, 24, 11108–19, 2004. With permission. [C] From Namiki, S., and Kanzaki, R., *Front. Neural Circuits*, 2, 1, 2008. With permission.)

2008; Figure 3.8). At higher frequencies (~20–50 Hz), odor-evoked responses in PNs exhibit synchronized activity (Laurent and Naraghi 1994; Heinbockel et al. 1998), which is thought to possess several computational roles such as improving olfactory discrimination and involvement in short-term memory in olfactory processing (Stopfer et al. 1997; Stopfer and Laurent 1999; Laurent 2002).

Christensen et al. (2000) have shown that a PN population can exhibit synchronized firing in response to odor stimulation. Using trains of brief stimulus pulses mimicking natural stimulus conditions, they have characterized several aspects of odor-evoked population activity in the moth AL (Christensen et al. 2003). First, odor presentation evokes a local field potential in the AL as well as in the MB, and these are not coherent. Second, the odor-evoked field potential is not globally coherent within the AL. Such spatial heterogeneity of synchronized activity has also been observed in the bumblebee AL (Okada et al. 2001).

## 3.5.4 GLOMERULUS-SPECIFIC LATERAL INTERACTIONS

Classically, the AL has been thought to perform sharpening of olfactory response profiles by lateral inhibition (Hildebrand and Shepherd 1997). In fact, the majority of the LNs have arborizations in

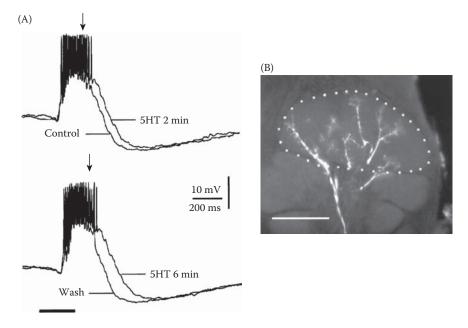
almost all glomeruli and show GABA-like immunoreactivity (Waldrop et al. 1987; Seki et al. 2008). However, recent electrophysiological, imaging, and modeling studies have revealed a more complex picture (Wilson et al. 2004; Linster et al. 2005; Silbering and Galizia 2007). In the silk moth AL, the response similarity between glomeruli is independent of the anatomical distance, suggesting the existence of distance-independent lateral interactions (Namiki and Kanzaki 2008). Reisenman et al. (2005) have found that input to the cumulus inhibits PNs innervating neighboring glomeruli, but input to these neighboring glomeruli does not inhibit PNs innervating the cumulus, indicating that lateral inhibition can be unidirectional. Subsequently, they have revealed that lateral inhibition originating from the cumulus is independent of the anatomical distance of the glomeruli it affects (Reisenman et al. 2008).

The mechanism of distance-independent inhibition is unknown, but two solutions are proposed. First, heterogeneous LNs connecting specific subsets of glomeruli may implement distance-independent inhibition (Matsumoto and Hildebrand 1981; Christensen et al. 1993; Seki and Kanzaki 2008; Reisenman et al. 2008). Second, passive membrane properties of LN dendrites probably enable local interactions (Christensen et al. 2001; Yamasaki et al. 2006). Subthreshold input to local compartments of LN dendrites may not propagate beyond a limit distance, and thus become a substrate of local interaction among specific subsets of glomeruli innervated by these LNs. In addition to inhibitory interactions, the presence of lateral excitatory action has been shown in the fly AL (Olsen et al. 2007; Shang et al. 2007). Recently, local circuit processing in the fly AL has been intensively investigated utilizing genetics. Chapter 2 discusses this topic in greater detail.

#### 3.5.5 NEUROMODULATION AND PLASTICITY IN THE ANTENNAL LOBE

Physiological experiments have shown that neural responses to pheromone and electrical stimuli in the moth AL are enhanced by serotonin (Kloppenburg and Hildebrand 1995; Kloppenburg et al. 1999; Hill et al. 2003; Figure 3.9). Serotonin also has effects on field potential oscillations (Kloppenburg and Heinbockel 2000) and is thus likely to affect olfactory information encoding. In PNs and possibly other AL neurons, the basis of such changes is the reduction of potassium currents upon exposure to serotonin application (Mercer et al. 1995; Kloppenburg et al. 1999). At AL circuit level, 5-hydroxytryptamine (5HT) increases sensitivity and improves ensemble discrimination of odors (Dacks et al. 2008). The ALs of B. mori and M. sexta contain a bilaterally symmetrical serotonergic neuron that innervates all glomeruli (Kent et al. 1987; Hill et al. 2002), mostly making output synapses (Sun et al. 1993). This neuron, similar to 5HT neurons with overall glomerular innervation patterns in other insects (Schürmann and Klemm 1984; Rehder et al. 1987; Breidbach 1990; Salecker and Distler 1990; Dacks et al. 2006), is the likely natural source for modulation by 5HT in the AL reflected in behaviorally determined sensitivity changes (see Section 3.2.3.2). It displays no odorant-specific response and shows slow, regular activity (Hill et al. 2002). It is a possible neural substrate for short-term and circadian modulation of olfactory sensitivity, but also for longterm structural changes (Kloppenburg and Mercer 2008). Juvenile hormone-dependent maturation of the AL also occurs at a longer timescale, increasing the sensitivity of AL neurons (Anton and Gadenne 1999). Interestingly, the age-dependent increase of sensitivity is specific to pheromoneresponsive neurons (Greiner et al. 2002). At the timescale of ongoing olfactory processing, nitric oxide is involved in shaping AL responses (Wilson et al. 2007). While other neuroactive substances have been identified in the AL of M. sexta (Homberg and Müller 1999; Dacks et al. 2005), B. mori (Iwano and Kanzaki 2005), and H. virescens (Berg et al. 2007), their roles in the modulation of information flow through the AL have, so far, not been investigated. Octopamine possibly plays a similar role in learning as in the honeybee because a neuron similar to the bee's putatively octopaminergic VUMmx1 (Hammer 1993; Schröter et al. 2007) has been found in H. virescens (Rø et al. 2007).

In AL neurons of male moths, different forms of central nonassociative plasticity have been demonstrated. Responses to pheromone are decreased for a brief period after mating, reflecting similar



**FIGURE 3.9** Modulation of pheromone-induced responses in AL projection neurons of male *Manduca sexta* by 5HT. (A) Whole cell recording of an MGC projection neuron under current clamp (hyperpolarizing current injection). Responses to antennal pheromone stimulation (duration marked by horizontal bar at bottom) before (control), during (2 and 6 min), and after (wash) application of 5-HT ( $10^{-4}$  M) are shown. The last spikes of the control and wash traces are indicated by arrows. (B) Morphology of the arborizations of the recorded neuron in the cumulus (dotted line) of the MGC. Scale bar: 50 µm. (From Kloppenburg, P., Ferns, D., and Mercer, A. R. *J. Neurosci.*, 19, 8172–81, 1999. With permission.)

changes in behavior (Gadenne et al. 2001; see Section 3.2.3.3). On the other hand, brief pre-exposure to pheromone was shown to enhance sensitivity at behavioral and AL physiological level (Anderson et al. 2007). Pavlovian conditioning, the pairing of an odor with a gustatory reward stimulus, is also capable of altering information processing in the AL. Notably, the number of neurons responsive to the conditioned odor stimulus increases as a result of learning (Daly et al. 2004).

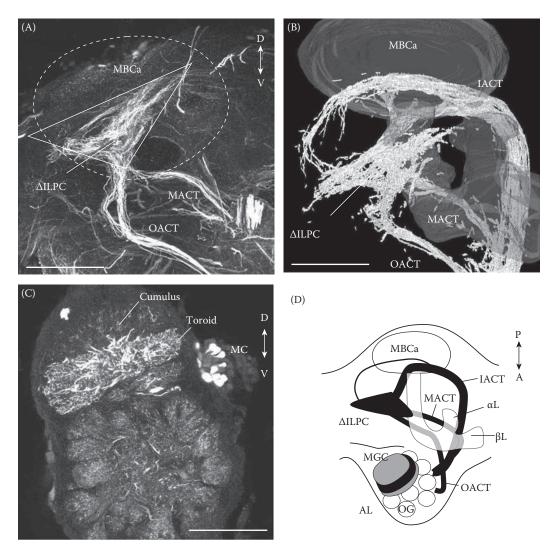
## 3.6 PROTOCEREBRAL OLFACTORY CIRCUITS

The outputs of the AL, the PNs, project to different PC targets. A wide target area of PNs is the LPC (Homberg et al. 1988; Kanzaki et al. 1989, 2003; Rø et al. 2007). Each PN has a single axon that innervates one of the ACTs. In *M. sexta*, five ACTs have been identified: the inner (IACT), outer (OACT), middle (MACT), dorsal (DACT), and dorso-median (DMACT) ACTs (Homberg et al. 1988; Kanzaki et al. 1989). Depending on the species, not all of these were found in other moths (Kanzaki et al. 2003; Rø et al. 2007). Some OACT projections have been found to the contralateral PC (Homberg et al. 1988; Wu et al. 1996).

Most PNs with axons in the IACT appear to have uniglomerular dendritic arborizations in the AL of moths (Christensen and Hildebrand 1987; Homberg et al. 1988; Kanzaki et al. 1989; Christensen et al. 1991; Anton and Homberg 1999; Rø et al. 2007; Anton and Hansson 1994, 1995), but some multiglomerular PNs running through the IACT exist (Hansson et al. 1991; Heinbockel et al. 2004). Uniglomerular PNs have also been found in the OACT and DMACTs (Anton and Homberg 1999; Homberg et al. 1988; Kanzaki et al. 2003). Putatively GABAergic PNs with multiglomerular dendritic arborizations and somata in the lateral cell cluster (LC) of the AL project in the MACT (Hoskins et al. 1986; Anton and Homberg 1999; Iwano and Kanzaki 2005). Some PNs of the LC projecting into the MACT are immunoreactive for FMRFamide (Iwano and Kanzaki 2005). In the

silk moth, the somata of uniglomerular MGC PNs are located in the medial cell cluster (MC) and their axons innervate the IACT, whereas the somata of multiglomerular MGC PNs are located in the LC and their axons run through the MACT or OACT (Kanzaki et al. 2003).

The segregation of the pheromone and general odor systems is maintained in the LPC as PNs from the ordinary glomeruli project to the LH, while PNs from the MGC project to a separate area in the ILPC (Homberg et al. 1988; Kanzaki et al. 1989). In *B. mori*, MGC PNs specifically innervated a circumscribed pyramidally shaped projection area between the LH and the MB calyx (MBCa), called the delta area of the inferolateral protocerebrum ( $\Delta$ ILPC), in which the projections representing the blend components occupy partially overlapping regions (Seki et al. 2005; Figure 3.10). PNs also project to the MBCa, where projections cover a substantial area of the calyx with wide axonal arbors. Projections from the MGC toroid subdivision, responsive to the major



**FIGURE 3.10** Innervation of MGC PNs in the LPC. (A) Anti-cGMP immunostaining in the LPC, showing axonal projections of PNs that define the  $\Delta$ ILPC. (B) Three-dimensional reconstruction of the data shown in (A) with the ACTs. (C) Anti-cGMP immunostaining in the AL. The label is largely restricted to MGC PNs innervating the toroid. (D) Schematic diagram of axonal projections of MGC PNs. Axonal projections from the toroid are mainly found in the  $\Delta$ ILPC. Scale bars: 100 µm. (From Seki, Y., Aonuma, H., and Kanzaki, R. *J. Comp. Neurol.*, 481, 340–51, 2005. With permission.)

pheromone blend component, only project to a restricted area of the MBCa (Kanzaki et al. 2003; Seki et al. 2005). The segregation of general odor and pheromone systems may be a general feature in insects (Jefferis et al. 2007).

The nature of information processing by PC neurons remains largely obscure. One particular problem is the fact that besides some easily recognizable structures, a sizable portion of the PC lacks clear compartments. High blend specificity and sophisticated multimodal response properties have been found in PC neurons (Kanzaki et al. 1991a, 1991b; Light 1986). However, complex response properties, in particular blend-specific responses, may be less common than expected and temporal resolution rather declines compared to AL PN neurons (Lei et al. 2001). Systematic work has so far only been done in two protocerebral areas, the MB and the LAL.

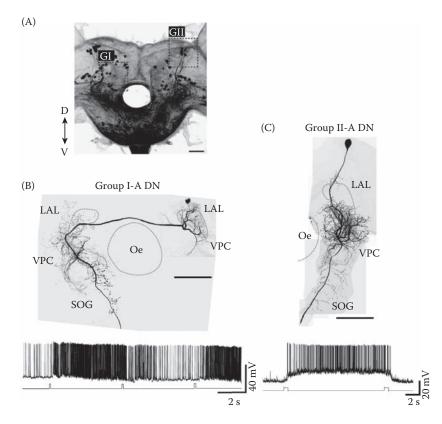
## 3.6.1 MUSHROOM BODY

While the MBs are a main recipient of odor information provided by AL PNs, little is known about their physiology in moths. The MBs are composed of Kenyon cells (KCs), but also contain the projections of AL PNs and other extrinsic neurons. The processes of the KCs form the MB pedunculi, lobes, and calyces, the latter being the input area. Due to their conspicuous structure and the convergence of multiple sensory modalities, the MBs have long been regarded as one of the highest centers in insect brains and are linked to associative learning and memory (Strausfeld et al. 1998; Heisenberg 2003; Farris 2005; Wessnizer and Webb 2006). The architecture of moth MBs has been investigated with different morphological methods, showing conspicuous subdivisions. In moths, the number of KCs is comparatively small, but they are unusually large and belong to morphological classes associated with the MB lobe subdivisions (Pearson 1971; Sjöholm et al. 2005, 2006; Sinakevitch et al. 2008; Fukushima and Kanzaki, 2009). The MB subdivisions are obvious also at the level of the pedunculus and may be interpreted in relation to input and output segregation, in particular considering the spatially restricted AL pheromone-sensitive PN projections in the calyx (see above). Odor information is represented by a sparse code in MB KCs of *M. sexta* (Ito et al. 2008). While a link between learning and the activity of MB KCs has been attempted, no temporal overlap of KC activity and reinforcement was found. Therefore, Hebbian plasticity in KCs seems unlikely to underlie the olfactory classical conditioning observed at behavioral level (Ito et al. 2008). Little is known about MB extrinsic cells in moths. One better-studied example of this class of neurons is the 5HT AL neuron, providing feedback from the MB and other PC areas (see Section 3.5.5).

#### 3.6.2 LATERAL ACCESSORY LOBE

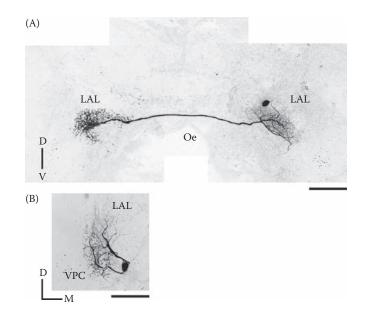
Based on the fact that descending neurons (DNs) show pheromone responses, the LAL of the PC and adjacent areas of the ventral protocerebrum (VPC) could be identified as important olfactory neuropils in which the output of the brain in response to olfactory stimuli is generated (Olberg 1983; Kanzaki et al. 1994). In several insect species, DN responses to stimuli of different modalities and their role in the control of various behaviors have been investigated (see Okada et al. 2003). DNs in moths have so far chiefly been investigated with pheromone and visual stimuli, since these are important for odor source localization behaviors.

DNs showing phasic excitation, phasic inhibition, or long-lasting excitatory or inhibitory aftereffects following pheromone stimulation have been found in *M. sexta* and *B. mori* (Kanzaki and Shibuya 1986; Kanzaki et al. 1991b; Mishima and Kanzaki 1999; Wada and Kanzaki 2005). While *M. sexta* DNs without projections in the LAL showed only phasic pheromone responses, state-dependent activity and tonic modulation by light intensity was observed in pheromonesensitive LAL DNs (Kanzaki et al. 1991b). DNs with state-dependent activity found in *B. mori*, show conspicuous flipflop activity, switching between low and high firing rates upon subsequent pheromone stimuli, reminiscent of a toggle flipflop circuit (Figure 3.11; Olberg 1983; Kanzaki et al. 1994; Mishima and Kanzaki 1999; Wada and Kanzaki 2005). There are two classes of flipflop



**FIGURE 3.11** Flipflopping descending neurons in the silk moth brain. (A) Distribution of DN somata in the brain of *Bombyx mori* labeled by backfilling through the neck connective. (B) Physiological responses to bombykol pulses (lower trace) and morphology of a Group-I DN. (C) Physiological responses to bombykol pulses (lower trace) and morphology of a Group-II DN. Scale bar: 100 µm. ([A] and [C] From Wada, S. and Kanzaki R., *J. Comp. Neurol.*, 484, 168–82, 2005. With permission. [B] From Mishima, T. and Kanzaki, R., *J. Comp. Physiol.* A, 183, 273–82, 1998. With permission.)

DNs on each side of the neck connective, firing in antiphase relative to the same class on the contralateral side and to the other class on the ipsilateral side (Kanzaki et al. 1994; Kanzaki and Mishima 1996). The flipflop DNs belong to two soma clusters containing pheromone-sensitive DNs (Kanzaki et al. 1994; Mishima and Kanzaki 1999; Wada and Kanzaki 2005), one located near the anterior border between DC and PC (Group I, three neurons with bilateral projections, one capable of flipflop responses) and another located anterodorsally just medial of the AL (Group II, 10–15 unilaterally confined cells of three morphological types, two of which can show flipflop responses). DNs are often multimodal. In Lymantria dispar DNs, synergistic effects between responses to moving patterns and pheromone, as well as responses only occurring in combined stimulation, were shown (Olberg and Willis 1990). Such responses are possible substrates of optomotor responses involved in upwind flight. In B. mori, flipflopping could be induced by light intensity changes or modulated by absolute light intensity as well as pheromone concentration, and some flipflop DNs also responded with graded responses to mechanical and moving visual stimuli (Olberg 1983; Kanzaki et al. 1994). One target of at least some flipflop DNs are motor neurons of the SOG controlling head movements that are correlated with turns in locomotion (Kanzaki and Mishima 1996; Mishima and Kanzaki 1998, 1999; Wada and Kanzaki 2005). The flipflop DNs have been implicated to represent command neurons controlling walking direction, thus being the neural substrates of zigzag walking, the main element of pheromone orientation behavior in B. mori.



**FIGURE 3.12** Morphology of LAL interneurons in the silk moth brain. (A) Morphology of a LAL bilateral interneuron. The neuron has smooth processes in the ipsilateral LAL and varicose process in the contralateral LAL, and responds to bombykol with lasting excitatory firing. (B) Morphology of a LAL local interneuron responsive to bombykol. The neuron innervates the ipsilateral LAL and adjacent VPC. Scale bar: 100 µm.

Besides DNs, the LAL/VPC region also contains LNs that are unilaterally confined and bilateral neurons (BNs), identified both in *B. mori* and *M. sexta* (Figure 3.12; Kanzaki et al. 1991a; Kanzaki and Shibuya 1992; Mori et al. 1999; Iwano et al. 2009). These neurons showed transient excitation or excitatory after-effects to ipsilateral pheromone stimulation and multimodal properties. The BNs, in particular, are thought to be important in the generation of flipflop activity by providing contralateral inhibition (Kanzaki et al. 1994) and some of them have been shown to be GABA-immunoreactive (Iwano et al. 2009).

A third, broadly defined class, are mostly unilateral interneurons linking the LAL and adjacent VPC neuropil with other protocerebral areas. A few neurons that establish direct connections between  $\Delta$ ILPC and LAL have been identified in *M. sexta* and *A. segetum* (Kanzaki et al. 1991a; Lei et al. 2001). Preliminary results from our laboratory taken together with the results of Lei et al. (2001) imply that pheromone information is largely relayed though an area in the superior median protocerebrum (SPMC). Other interneurons connect the LAL with the MB and show features similar to MB extrinsic cells in other insects, including after-effects (Kanzaki et al. 1991a).

## 3.7 OUTLOOK

Research in moth olfaction has come a long way since the isolation of the first sex pheromone (Butenandt et al. 1959) and the discovery of the electroantennogram (EAG; Schneider 1957), still the most widely used technique to assess ORN responses in insects. EAGs are now being used to create highly specific sensors for odorants (Park et al. 2002), and the fact that insect odorant receptors are capable of direct transduction into electrical signals (see Section 3.3.4.3) has great potential for use as odorant sensors in measurement apparatus. Learning in moths and other insects is employed to use insects for locating odor sources of interest at various spatial scales (Rains et al. 2008). Pheromone research and host-plant-induced behaviors remain highly active fields in moth olfaction not least because of the economic importance of a number of moth species as agricultural pests in still widely used large-scale monocultures. However, pheromone-induced responses are

also a tool in basic research with applications in engineering, for instance in autonomous systems, because they are one of the most accessible approaches to study mechanisms of reliable, robust odor source localization, while allowing precise control over perturbations due to the high specificity of pheromone-induced behavioral programs. The first implementations have started to appear in the area of odor source localization as hybrid robots, coupling biological information processing to artificial effectors (Emoto et al. 2007; Kanzaki et al. 2008).

In this field, the elucidation of protocerebral mechanisms generating the steering control outputs relayed by DNs is currently a major challenge. The relatively small size of moth brains and a large body of identified neuron data are now being used to attempt rebuilding behaviorally relevant circuits of the moth brain by means of realistic biophysical simulations. Genetic manipulations have become feasible and the silk moth (*B. mori*) in particular, being flightless and showing locomotion only in response to stimulation, is a very convenient and safe system in which the full array of these techniques may be applied (Yamagata et al. 2008).

The general odor detection system of moths also holds promises for the future. It will be a valuable tool in understanding odorant information encoding in the CNS, especially in conjunction with learning paradigms that have recently been developed, allowing direct evaluation of neural activity through behavioral performance. Outside the more reductionist laboratory setting, research in moth olfaction is increasingly linking field conditions with their multimodal stimulus conditions to behavior and neurobiology, leading to a better understanding of how mechanisms evolved as adaptations to environmental constraints.

### ACKNOWLEDGMENTS

We would like to thank Sylvia Anton, Uwe Homberg, Sid Simon, and our colleagues in the laboratory, in particular, Douglas Bakkum and Ryota Fukushima, for their help in improving the manuscript.

## ABBREVIATIONS

5HT:	5-hydroxytryptamine (serotonin)
A:	anterior
ABPX:	antennal binding protein X
ACT:	antenno-cerebral tract
AL:	antennal lobe
AMMC:	antennal mechanosensory and motor center
AN:	antennal nerve
AOTu:	anterior optic tubercle
BN:	PC bilateral neuron
CC:	central complex
$\Delta$ ILPC:	delta area of the inferolateral protocerebrum
D:	dorsal
DACT:	dorsal antenno-cerebral tract
DC:	deutocerebrum
DMACT:	dorso-median antenno-cerebral tract
DN:	descending neuron
EAG:	electroantennogram
FF:	flipflop neuron
GABA:	γ-aminobutyric acid
GOBP:	general odorant-binding protein
GPCR:	G-protein-coupled receptor
IACT:	inner antenno-cerebral tract
IP <sub>3</sub> :	inositol-(1,4,5) trisphosphate

L:IateralLAL:lateral accessory lobeLDN:labial nervesLC:lateral cell cluster of the ALLH:lateral nornLN:AL local interneuronLo:lobulalobl:MB lobeletLPC:lateral protocerebrumM:medialMACT:middle antenno-cerebral tractMB:mushroom bodyMBCa:MB calyxMBL:MB lobesMBPe:MB pedunculusMBYT:MB Y-tractMC:medial lobeOACT:outer antenno-cerebral tractOBP:odorant-binding proteinODE:odorant-binding proteinODE:odorant degrading enzymeOe:esophagus (esophageal foramen)OL:optic lobeOR:olfactory receptor (protein)ORN:olfactory receptor neuronP:posteriorPBP:pheromone-binding proteinPDE:pheromone degrading enzymePER:proboscis extension responsePIP_2:phosphatidyl inositol 4,5-bisphosphatePN:AL projection neurons:sensillum/sensillaSMPC:superior median protocerebrumSOG:subesophageal ganglionTC:tritocerebrumV:ventralV:ventralV:ventralV:ventralVPC:ventral protocerebrum	KC:	Kenyon cell
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vL: MB vertical lobe	TC:	tritocerebrum
	V:	ventral
VPC: ventral protocerebrum		
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# 4 Olfactory Coding in Larvae of the African Clawed Frog *Xenopus laevis*

Ivan Manzini and Detlev Schild

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	Anato: 4.2.1 4.2.2 Transd 4.3.1 4.3.2 4.3.3 Odor-7 in the 4.4.1 4.4.2 4.4.3 Modul 4.5.1 4.5.2	<ul> <li>4.2.1 Principal Cavity (PC) and Vomeronasal Organ (VNO)</li></ul>

The sensing of molecules in the environment is critical to the survival of every organism. It is, therefore, hardly surprising that most animals have developed highly sophisticated olfactory systems. In contrast to other sensory systems, large portions of the genome are devoted to encode the receptors of this sensory system. The past years have seen an explosion in studies aimed at understanding the functioning of the olfactory system. These studies cover all levels of analysis—from genes to behavior. Considerable progress has been made in understanding the molecular organization of all stages of the olfactory pathway. Consequently, particular effort was spent on the question of how information contained in odorant molecules is encoded and processed at the various levels of these studies focused on adult animals, the state of knowledge of the embryonic or larval development of olfactory systems are inherently difficult and so far have not been carried out. In this respect, amphibians are particularly suited. Their fertilized eggs develop into free-swimming larvae before metamorphosing into juvenile animals. Ontogenetic stages of various amphibians are well characterized and easy to handle. This chapter aims to present a detailed overview of the

current knowledge of the organization and function of the olfactory system of a premetamorphotic amphibian, the african clawed frog *Xenopus laevis*.

## 4.1 INTRODUCTION

Various amphibian species, including *X. laevis*, have been adopted as experimental animal models in numerous studies dealing with the function of the olfactory system, and numerous papers have been published about amphibian olfaction (for reviews, see Eisthen 1997, 2002; Schild and Restrepo 1998; Jørgensen 2000; Kauer 2002; Ache and Young 2005). Therefore, in this chapter, we do not describe general features of the amphibian olfactory system, but rather focus on specific new data that diverge from the current view of olfactory coding and possibly provide new insights in how the olfactory system develops.

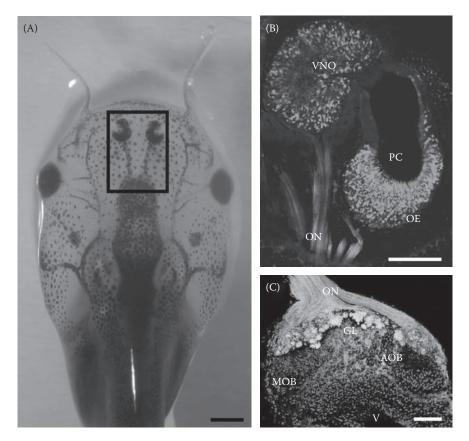
# 4.2 ANATOMY AND CELLULAR ORGANIZATION OF THE OLFACTORY SYSTEM OF LARVAL XENOPUS LAEVIS

### 4.2.1 PRINCIPAL CAVITY (PC) AND VOMERONASAL ORGAN (VNO)

Larval X. *laevis* (Figure 4.1A) have two distinct olfactory organs: the principal cavity (PC) and the vomeronasal organ (VNO; Figure 4.1B; Hansen et al. 1998). These two olfactory organs, like those of other vertebrates (see also Chapters 5 and 6), originate from paired olfactory placodes that first become distinguishable from the surrounding ectoderm at stage 23 (stage classification according to Niewkoop and Faber 1994), and slightly later begin to invaginate to form the olfactory pits. At about stage 40, the olfactory pits start to segregate into PC and VNO (Föske 1934; Niewkoop and Faber 1994). At stage 51–52, a second cavity, the middle cavity, becomes apparent. During metamorphosis, the middle cavity strongly expands and the PC is reorganized into the adult PC. In adult *X. laevis*, these two cavities together with the VNO form the tripartite olfactory organ of the adult frog (Altner 1962; Föske 1934; Burd 1991; Higgs and Burd 2001; Reiss and Burd 1997a, 1997b; Hansen et al. 1998).

As in other amphibians, the olfactory receptor (OR) gene repertoire of *X. laevis*, in several respects, represents an intermediate between fish and terrestrial vertebrates (Niimura and Nei 2005; Shi and Zhang 2007; Saraiva and Korsching 2007). *X. laevis* has an OR repertoire of several hundred genes (Niimura and Nei 2005) and a large vomeronasal receptor (V1R and V2R) repertoire exceeding even that of rodents (Niimura and Nei 2005). ORs closely related to fish OR and those closely related to mammalian OR (see Chapter 7) are both expressed in the larval PC (Freitag et al. 1995; Mezler et al. 1999). After metamorphosis, "fishlike" ORs are expressed solely in the middle cavity and "mammalianlike" OR only in the adult PC (Mezler et al. 1999). In adult *X. laevis*, the PC is filled with air and serves as "air nose," the middle cavity is filled with water and serve as "water nose" (Altner 1962). At least in larval *X. laevis*, V2Rs are almost exclusively expressed in the VNO (Hagino-Yamagishi et al. 2004). In *X. tropicalis*, V1Rs have been shown to be expressed predominantly in the larval PC and not in its VNO (Date-Ito et al. 2008). The VNO is filled with water throughout the animals' life (Altner 1962).

As in other vertebrates, the olfactory epithelia of the larval *X. laevis* PC and VNO are made up of three main cell types: olfactory receptor neurons (ORNs) that transmit the olfactory information from the nose to the olfactory bulb (OB) in the brain, sustentacular cells (SCs) that share properties with both glial and epithelial cells, and basal cells (BCs), including olfactory stem cells, which maintain the regenerative capacity of the olfactory epithelium (OE; Graziadei and Metcalf 1971; Graziadei 1971, 1973; Hansen et al. 1998). From stage 50 on, the OE in the PC contains two types of ORNs, ciliated and microvillar, which appear to be distributed randomly within the OE (Hansen et al. 1998). At these late stages, the PC also contains two types of SCs, one type having short microvilli and containing secretory vesicles in the apical part, and another



**FIGURE 4.1** (See color insert following page 206.) The main and accessory olfactory system of larval *Xenopus laevis*. (A): Larval *Xenopus laevis* (stage 51) The black rectangle outlines the first two stages of the olfactory system (scale bar 2 mm). (B): Horizontal overview over the olfactory epithelium and the vomeronasal organ (VNO, vomeronasal organ; PC, principal cavity; OE, olfactory epithelium; ON, olfactory nerve). The neurons were backfilled through the olfactory nerve using biocytin/avidin staining (green fluorescence) (scale bar 200  $\mu$ m). (C): Horizontal overview over the olfactory bulb (ON, olfactory nerve; MOB, main olfactory bulb; AOB, accessory olfactory bulb; GL, glomerular layer; V, ventricle). Biocytin injection into the olfactory nerve (green fluorescence), synaptophysin immunostaining (red fluorescence), and DAPI nucleic acid staining (blue fluorescence) (Scale bar 100  $\mu$ m). ([C] modified from Nezlin et al. *J. Comp. Neurol.*, 464, 257–68, 2003.)

type with kinocilia and no vesicles (Hansen et al. 1998). In the basal portion of the OE, close to the lamina propria, BCs can be found (Hansen et al. 1998). The larval PC has no Bowman glands (Hansen et al. 1998). The larval VNO has only one type of receptor cell bearing microvilli and one type of SC bearing kinocilia. The BCs show the same characteristics as those of the PC epithelium (Hansen et al. 1998).

# 4.2.2 MAIN AND ACCESSORY OLFACTORY BULB (OB)

The organizational principles of the main and accessory olfactory bulb (MOB and AOB; Figure 4.1C) are conserved in different species across phyla (Hildebrand and Shepherd 1997; Rössler et al. 2002; Lledo et al. 2005). From the surface to the center of the OB, there are six discernible layers: the nerve layer, the glomerular layer (GL), the external plexiform layer (EPL), the mitral cell layer (MCL), the internal plexiform layer (IPL), and the granule cell layer (GCL). Axon terminals of ORNs of the PC synapse directly onto second-order neurons in the OB, forming spheroidal structures

called glomeruli. In the premetamorphotic stages of *X. laevis*, the GL of the MOB is subdivided into a ventral and a dorsal part (Fritz et al. 1996; Nezlin and Schild 2000). The ventral MOB has clearly discernible glomeruli, while the dorsal MOB does not. Instead, the GL of the dorsal MOB consists of an apparently structureless fiber meshwork with some embedded fiber aggregations. About 200 periglomerular cells (PGC) reside in the GL and the EPL (Nezlin and Schild 2000). In contrast to the GL in mammals (Pinching and Powell 1971; Chao et al. 1997), where periglomerular cells (PGCs) form a wall around every glomerulus, but similar to the zebrafish (Byrd and Brunjes 1995), the glomeruli of larval *X. laevis* are not surrounded by cell bodies of PGC or by glia cells (Nezlin and Schild 2000; Nezlin et al. 2003). In the MOB of larval *X. laevis*, there are about 350 glomeruli with diameters in the range of  $10-40 \mu m$  (Nezlin and Schild 2000; Manzini et al. 2007b).

The spatial distribution of glomeruli in the MOB revealed that the GL of the MOB of larval *X. laevis* is organized in at least four spatially distinct clusters: a lateral, intermediate, medial, and an additional very small cluster, situated in the very ventral part of the MOB (Manzini et al. 2007b). The lateral, intermediate, medial, and small cluster consist of about 175, 70, 100, and five glomeruli, respectively (Manzini et al. 2007b). Another work reports that ORN axons of larvae of identical stages terminate in up to nine different projection fields in the OB (Gaudin and Gascuel 2005). The higher number of clusters/projection fields in the work by Gaudin and Gascuel (2005) is explained by the fact that they performed a more detailed subdivision of the four bigger clusters identified by Manzini et al. (2007b).

The estimates of the number of mitral cells (MCs) in the MOB of stage 54 larvae range from about 2000 (Nezlin and Schild 2000) to about 20,000 (Byrd and Burd 1991). As Byrd and Burd counted all nuclei in the MCL/EPL, including glia and developing neurons, their number is certainly an upper estimate for the number of MCs. As the MC number obtained by Nezlin and Schild (2000) is based on backtracing from the lateral olfactory tract (LOT), it is certainly a lower limit. Granule cells (GC) of both the MOB and the AOB appeared as a compact group of cells near the paraventricular ependyma.

Axons of vomeronasal receptor neurons (VRNs) project to the AOB. The AOB is situated lateroventrally with respect to the MOB. In the AOB, the MCL is in immediate proximity to the GL, leaving only a very narrow EPL (Nezlin and Schild 2000). The glomeruli of the AOB are smaller and more densely packed than those of the MOB (Figure 4.1B). About 350 glomeruli, approximately 70 PGC, and 2500 MC have been estimated in the AOB (Nezlin and Schild 2000). Axons of MCs of MOB and AOB form the LOT and project to higher olfactory centers. How exactly MCs are connected to these is still not known.

As to the spatial propagation, first, from ORNs to glomeruli and second, from MCs to higher brain regions, a remarkable parallelization can be observed. First, individual olfactory receptor neuron (ORN) axons, as identified by dye injection into individual ORNs, bifurcate several times before entering a small number of glomeruli (2 or 3; see also Section 4.4.2). The resulting action potential splitting could be important in that it introduces correlated inputs to glomeruli in the developing system (Nezlin and Schild 2005), i.e., subsequent action potentials delivered onto the same intraglomerular postsynaptic compartment stem from the same ORN in the larval stage and possibly from different ORNs of the same ORN class in the adult. Second, all MCs connected to the same glomerulus have been shown to be synchronous (Chen et al. 2009), i.e., identical MC activity copies are sent to higher brain regions, which may be crucial for odor recognition and memory formation.

# 4.3 TRANSDUCTION MECHANISMS IN OLFACTORY RECEPTOR NEURONS (ORNs)

#### 4.3.1 MAIN OLFACTORY SYSTEM

In terrestrial vertebrates, the vast majority of ORNs possess the canonical cAMP-mediated transduction pathway (see Chapter 8), but a few ORN subgroups have been shown to be endowed with alternative transduction cascades (Ma 2007; Breer et al. 2006; see also Chapter 9). In aquatic vertebrates, cAMP-independent transduction mechanisms appear to be more widespread (Ma and Michel 1998; Delay and Dionne 2002; Manzini et al. 2002b; Hansen et al. 2003; Manzini and Schild 2003). This is particularly evident in larval *X. laevis*. The main OE of larval *X. laevis* contains at least two subsets of ORNs with different transduction mechanisms and different odorant specificities (Figure 4.2A and B; Manzini et al. 2002b; Manzini and Schild 2003; Czesnik et al. 2006). One subset is activated by amino acid odorants in a cAMP-independent way (Manzini et al. 2002b; Manzini and Schild 2003; Czesnik et al. 2002b; Manzini and Schild 2003; Czesnik et al. 2006), while another subset responds to pharmacological agents activating the cAMP cascade. Bile acids and amines appear to be the natural odorants of this second subset of ORNs (Manzini I., unpublished data). At present, it is not known which transduction cascade is coupled to ORs sensitive to amino acids. The phospholipase C/IP<sub>3</sub>-mediated or the guanylyl cyclase D/cGMP-mediated cascades are putative candidates, but, to date, this question has not been answered. Whether the two ORN subgroups represent the two cytologically distinct ORNs (ciliated and microvillous; see Section 4.2.1.1) that have been shown to coexist in the larval PC, is not known.

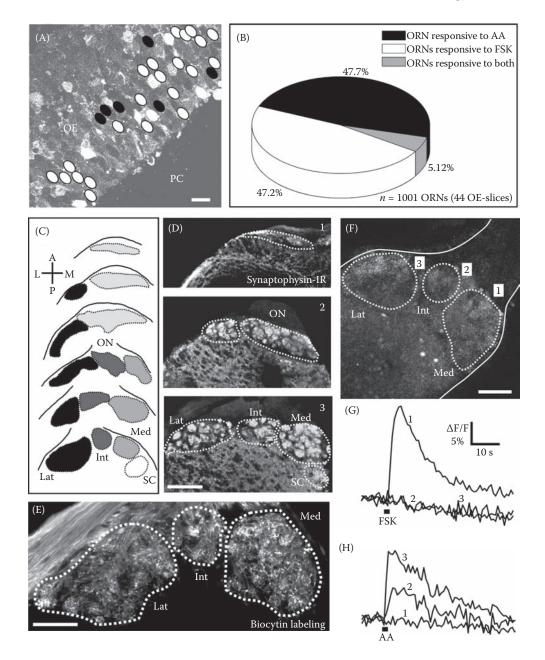
#### 4.3.2 DIFFERENT TRANSDUCTION MECHANISMS ESTABLISH FUNCTIONAL SUBSYSTEMS

The axons of the two abovementioned ORN subsystems differentially project to the glomerular clusters present in the MOB (Figure 4.2C through E; Manzini et al. 2002b, 2007b). The subset of ORNs lacking the cAMP-dependent transduction mechanism (responsive to amino acid) projects almost exclusively to glomeruli in the lateral MOB (belonging to the lateral glomerular cluster), and the subset of ORNs endowed with the cAMP transduction cascade (mainly responsive to bile acids and amines) exclusively project to glomeruli in the medial OB (medial glomerular cluster; Figure 4.2F through H). The existence of four to nine distinguishable glomerular clusters or projection fields (see Manzini et al. 2007b; Gaudin and Gascuel 2005) suggests that the main OE of larval *X. laevis* possibly contains more ORN subgroups than the two that have been identified so far. As to the subsets of ORNs that project to the intermediate and the small cluster, a functional definition is still lacking.

The synaptic terminals within the glomerular clusters in the lateral and medial MOB show clear differences in the expression of presynaptic proteins (Manzini et al. 2007b). The presynaptic vesicle protein, synaptophysin, and the presynaptic membrane proteins, syntaxin and SNAP-25, are uniformly distributed in the entire GL. Synaptotagmin, another presynaptic vesicle protein, known to function as a  $Ca^{2+}$  sensor for the regulated exocytosis of neurotransmitters, is expressed in the lateral and partly in the intermediate glomerular clusters, but it is missing in the medial cluster (Manzini et al. 2007b). The identity of the  $Ca^{2+}$  sensor in the medial and the small cluster is unknown. This inhomogeneity of presynaptic protein expression is an additional striking diversity, showing the relevant difference of the ORN subsystems of larval *X. laevis*. These diverse subsystems with different functional relevance (amino acid vs bile acid/amine odorants) possibly emerged at different points in the evolution of the olfactory system and certainly fulfill different olfactory requirements of the larvae.

#### 4.3.3 ACCESSORY OLFACTORY SYSTEM

In addition to the main olfactory system with its subsystems, larval *X. laevis* also have a functional accessory olfactory system. In an anatomical study, no apparent clustering of the glomeruli of the AOB has been noticed (Nezlin and Schild 2000). An electrophysiological study has shown that MCs and GCs of the AOB are spontaneously active in premetamorphotic larvae (Czesnik et al. 2001). The natural odorants/pheromones and the physiological role of the AOB in larval *X. laevis* remain to be determined.



**FIGURE 4.2** Functional subsystems within the main olfactory system of larval *Xenopus laevis*. A: Fluo4-AM-stained acute slice preparation of the olfactory epithelium (image acquired at rest; OE, olfactory epithelium; PC, principal cavity). The black and white ovals indicate ORNs of this slice that responded to a mixture of amino acids (AA) and to forskolin (FSK), respectively. Note that within this OE, there is no overlap between ORNs responding to the different stimuli (scale bar 20  $\mu$ m). B: Occurrences of correlated and uncorrelated responses to amino acids and forskolin of OE slices plotted as a pie chart (*n* = 1001 ORNs out of 44 acute OE slices). C: Schematic representation of the glomerular distribution in the MOB. The axes indicate: A, anterior; P, posterior; L, lateral; M, medial. Note that the glomeruli aggregate into glomerular clusters: int, intermediate glomerular cluster; lat, lateral glomerular cluster; med, medial glomerular cluster; sc, small glomerular cluster. D: Horizontal view of synaptophysin immunolabeled sections of the anterior part of the MOB at three different heights (D1, dorsal OB; D2, intermediate OB; D3, ventral OB) clearly show the glomerular clusters schematized in C (scale bar 100  $\mu$ m). E: Horizontal section of the MOB anterogradely labeled with biocytin

# 4.4 ODOR-TUNING PROPERTIES OF OLFACTORY RECEPTOR NEURONS (ORNs) AND WIRING SPECIFICITY IN THE OLFACTORY SYSTEM

Following the discovery of the OR gene family (Buck and Axel 1991), a multitude of studies have been carried out to understand how ORNs express specific ORs and how these ORNs are connected to the OB (e.g., Ressler et al. 1994; Vassar et al. 1994; Treloar et al. 1996; Feinstein and Mombaerts 2004; Mombaerts 2006). From these studies, two basic principles of olfactory coding have emerged. In adult mammals, each ORN expresses one type of OR (Nef et al. 1992; Strotmann et al. 1992; Ressler et al. 1993; Vassar et al. 1993; Chess et al. 1994; Malnic et al. 1999; Mombaerts 2004, 2006; see also Chapter 7), and all ORNs that express the same OR form a class of sensory neurons and project a single unbranched axon to a single or a few glomeruli within the OB (Ressler et al. 1994; Vassar et al. 1994; Mombaerts, 1996, 2006; see also Chapter 5). These features are considered the morphological basis of chemosensory maps connecting receptor specificities to the neuronal network of the OB.

In adult mammals also the second order neurons of the OB, MCs and tufted cells (TCs), follow a characteristic glomerular innervation pattern. Each MC sends a single primary dendrite to a single glomerulus (Shipley and Ennis 1996). In addition to this primary dendrite, each MC has several secondary dendrites that protrude into the EPL, often covering large OB territories. In contrast, MCs in the turtle typically send two primary dendrites into two glomeruli (Mori et al. 1981), showing that striking differences exist between higher and lower vertebrates. Mammalian TCs often feature more apical dendrites that innervate a number of glomeruli. Similar to MCs, TCs also have secondary dendrites that extend in the EPL (Shipley and Ennis 1996). In lower vertebrates, the distinction between MCs and TCs is not as clear as in higher vertebrates. Therefore, in the following, we often use the term MCs in the sense of mitral/TCs herein.

# 4.4.1 EXPRESSION OF MULTIPLE OLFACTORY RECEPTOR (OR) TYPES IN OLFACTORY RECEPTOR NEURONS (ORNS) OF LARVAL XENOPUS LAEVIS?

Several recent findings suggest that in larval *X. laevis*, a subset of ORNs express more than one type of OR. A recent study, where response profiles of individual ORNs to 19 amino acids were recorded, showed that 204 out of 283 ORNs responded differently to these stimuli (Manzini and Schild 2004). Accordingly, in the OE of larval *X. laevis*, there are at least 204 classes of ORNs differentially tuned to 19 amino acid odorants. Explaining such a high diversity of ORN classes by assuming one OR-type per ORN would imply the existence of a minimum of 200 ORs tuned to amino acid odorants. As there are 410 ORs in the *X. tropicalis* genome (Niimura and Nei 2005) and most probably a similar number also in *X. laevis*, it appears rather unlikely that at least 200 of them are more or less broadly and differentially tuned to amino acid odorants. The 19 amino acids could unambiguously be detected if there were 19 classes and the ORNs of each class would detect exactly one amino acid. Obviously, as few as five classes might code for 19 amino acids in case these would respond with appropriate combinations of their activities. As 10 ORN classes have been shown to be very specifically tuned to just one out of the 19 amino acids used (Manzini and Schild 2004), the first assumption appears to be more plausible. In the same study, it was

**FIGURE 4.2** (Opposite) through the olfactory nerve also depicts the three main glomerular clusters of the MOB (scale bar 100  $\mu$ m). F: Fluo-4-stained OB of a nose-brain preparation (image acquired at rest). The dotted lines indicate the approximate borders of the three main glomerular clusters of the MOB (1 = medial cluster; 2 = intermediate cluster; 3 = lateral cluster) (scale bar 100  $\mu$ m). G: Time courses of the [Ca<sup>2+</sup>]<sub>i</sub> responses of the three glomerular clusters upon mucosal application of forskolin (medial cluster: line 1; intermediate cluster: line 2; lateral cluster: line 3). H: Time courses of the [Ca<sup>2+</sup>]<sub>i</sub> responses of the three glomerular clusters upon mucosal application of amino acids (same labeling as in G). ([B] Modified from Manzini, I. and Schild, D., *J. Physiol.*, 551, 115–23, 2003. [C through G] Modified from Manzini, I. et al. *Eur. J. Neurosci.*, 26, 925–34, 2007b.)

observed that over ontogenetic stages, a narrowing of the response profiles of individual ORNs takes place. This analysis suggests that the abovedescribed high number of response profiles of individual ORNs could be a feature of the animals' ontogenetic stage. This hypothesis has been reinforced by a recent theoretical analysis of the 283 response profiles to amino acids (Schild and Manzini 2004).

It is generally accepted that all ORNs that express the same OR-type (ORN classes) project their axon to a single determined glomerulus within the OB. Therefore, it should be expected that individual glomeruli of the OB have response profiles identical to those of individual ORNs. In larval X. laevis, however, this appears not to be the case. Response profiles to amino acids of individual glomeruli clearly diverge from the response profiles recorded from ORNs. A thorough comparison of the response profiles of ORN glomeruli showed that individual amino acid-sensitive glomeruli tend to be tuned much narrower than ORNs (Manzini et al. 2007a). Furthermore, in contrast to the ORN response profiles, a narrowing of the glomerular response profiles over ontogenetic stages does not take place (Manzini et al. 2007a). So far, this is the only species where ORN and glomerular response profiles of a group of odorants have been compared (see also Figure 4.3). Taken together, the response profile data of ORNs and glomeruli allow the hypothesis that immature ORNs of X. laevis, i.e., not yet fully connected to the target glomeruli in the OB, express a number of amino acid-sensitive ORs and lose most of them after having found their target glomerulus and may finally express one OR. The PC (water nose) of tadpoles of lower stages obviously has more immature ORNs not yet connected to the OB as compared to animals of higher stages, where the premetamorphic water nose has fully developed.

#### 4.4.2 AXON TARGETING OF OLFACTORY RECEPTOR NEURONS (ORNS)

Atypically, in larval *X. laevis*, axons of individual ORNs mostly project to more than one glomerulus (Figure 4.4; Nezlin and Schild 2005). Only a minority of axons project into a single glomerulus. After entering the OB, ORN axons bifurcate into multiple axonal branches, which then typically innervate two or three glomeruli. Before entering a glomerulus, single axonal branches typically split again into sub-branches and enter the same glomerulus from opposite sides. Interestingly, the few axons that innervate only one glomerulus also split into two branches, which then project into the glomerulus from opposite sides. This means that in all cases, irrespective of the number of glomeruli innervated by one primary axon, not less than two axonal branches enter each glomerulus. It was even observed that axonal branches crossed the midline of the brain, entered the contralateral OB, bifurcated again, and innervated a glomerulus in the contralateral OB (Nezlin and Schild 2005).

This unexpected wiring allows several interpretations regarding its functional implication in the olfactory coding of larval *X. laevis*. The first one pertains to the chemosensory map, from the sensitivities of ORNs to the spatiotemporal activity pattern of OB neurons. This sensitivity-to-space map is presumably not as precise and focused in larval or embryonic stages as it is in adults. It is not known whether a similar axonal branching persists in adult *X. laevis*.

The atypically broad wiring properties could also be related to the abovedescribed response profile data of ORNs and glomeruli (Manzini and Schild 2004; Manzini et al. 2007a). Axons that project into more than one glomerulus could originate from broadly tuned immature ORNs, still in the process of defining their final selectivity and connecting to the respective glomerulus. In turn, the few axons that innervate only one glomerulus, could come from mature more narrowly tuned ORNs. However, this might imply that the synaptic contacts of the axons that project into more than one glomerulus are not yet fully functional, as glomeruli broadly tuned to amino acids apparently are not very numerous in the larval OB (Manzini et al. 2007a).

The fact that all axons, even those that innervate one glomerulus, split into sub-branches and enter the glomerulus from opposite sides is rather intriguing. What physiological implications

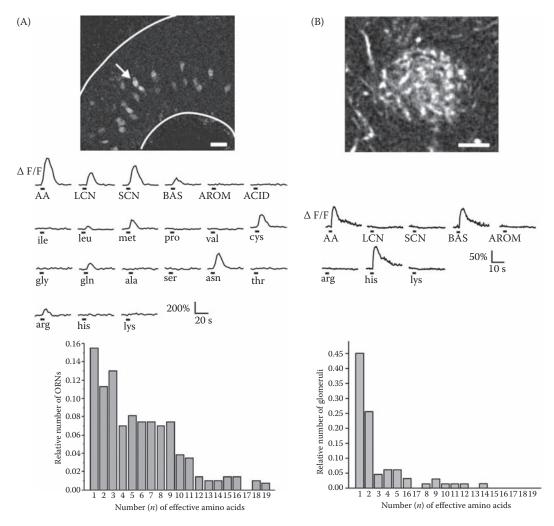
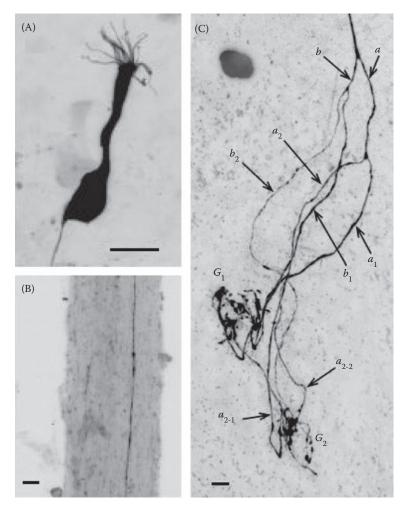


FIGURE 4.3 Response profiles to amino acids of individual olfactory receptor neurons and individual glomeruli of larval Xenopus laevis. A: ORNs of an acute slice preparation of the OE activated by application of a mixture of 19 amino acids. The ORN, indicated by an arrow, responded to the mixture of all 19 amino acids, the submixture of the long chain neutral amino acids (LCN), L-methionine, L-leucine, the submixture of the short chain neutral amino acids (SCN), L-cysteine, L-glutamine, L-asparagine, the submixture of the basic amino acid (BAS), and to L-arginine. No response to the submixtures of aromatic (AROM) or acidic (ACID) amino acids, nor to the remaining single amino acids of the responsive groups. The histogram shows the number of effective amino acids (n out of 19 amino acids) per ORN (n = 283 ORNs, 49 slices) (scale bar 20 μm). ([A] Modified from Manzini, I. and Schild, D., J. Gen. Physiol., 123, 99–107, 2004. B: Image of the fine intraglomerular structures of an amino acid activated glomerulus of a nose-brain preparation. The traces show the responses to the mixture of all amino acids, to the submixture of the basic amino acids, and to L-histidine. There was no response to the LCN, the SCN, and/or AROM amino acids. Additionally, the glomerulus did not respond to the remaining single amino acids of the BAS amino acid submixture. The histogram shows the number of effective amino acids (n out of 15 amino acids) per individual glomerulus (n = 67, 41 nose-brain preparations) (scale bar 10 µm). ([B] Modified from Manzini, I. et al. Eur. J. Neurosci., 26, 925-34, 2007b; for more information see also these papers.)

could this particular branching pattern have? From what is known about axonal action potential propagation, we can assume that odor-induced axon potentials generated in a particular ORN is duplicated at every bifurcation of the axon, so that in all cases multiple action potentials enter a glomerulus almost simultaneously. These specific projection patterns could increase the fidelity



**FIGURE 4.4** Most ORN axons of larval *Xenopus laevis* project into more than one glomerulus. A: High magnification image of a biocytin/avidin-stained ORN, showing the dendrite with apical cilia and the axon. The axon disappears into a deeper layer of the slice (scale bar 10  $\mu$ m). B: In the ON of the same preparation, the axon could be traced along the whole length of the ON (scale bar 10  $\mu$ m). C: Representative innervation pattern of two glomeruli by the same ORN. The ORN axon bifurcates into two branches, *a* and *b*, each bifurcating again into *a*<sub>1</sub> and *a*<sub>2</sub>, and *b*<sub>1</sub> and *b*<sub>2</sub>, respectively. The fibers *a*<sub>1</sub> and *b*<sub>1</sub> innervate glomerulus *G1*, and the branches *a*<sub>2</sub> and *b*<sub>2</sub> run into glomerulus *G2*. Before entering the glomerulus, the fiber *a*<sub>2</sub> divides into *a*<sub>2-2</sub> (scale bar 10  $\mu$ m). (Modified from Nezlin, L.P. and Schild, D., *J. Comp. Neurol.*, 481, 233–39, 2005.)

of transmission to the dendrite of a projection neuron and/or alternatively excite different projection neurons. The length differences of the sub-branches can be as big as  $60 \ \mu m$  (Nezlin and Schild 2005). The correlated action potentials must therefore arrive at their synaptic sites at slightly different times. The time delay between action potentials of the same bifurcation could be as large as  $500 \ \mu s$  (assuming  $50 \ \mu m$  length difference; Nezlin and Schild 2005). The temporally slightly displaced synaptic inputs could possibly enhance the synchronous activation of MCs within the target glomerulus. This might be particularly important considering that odorantinduced ORN firing rates are low in larval *X. laevis* (up to 20 Hz; Manzini et al. 2002a, 2002b). In higher vertebrates, synchronous activation of MCs has been put into relation with glutamate spillover in glial-wrapped subcompartments of the glomerulus (Schoppa and Westbrooke 2001). In contrast to higher vertebrates (Chao et al. 1997; Kasowski et al. 1999), glomeruli in larval *X. laevis* are not ensheathed by glial processes and most presumably do not include glial-wrapped subcompartments (Nezlin et al. 2003), which does not, however, preclude a few, but increasing number of PGCs. Axonal bifurcations could be an alternative way to ensure synchrony of the MCs of individual glomeruli.

# 4.4.3 GLOMERULAR WIRING SPECIFICITY OF SECOND ORDER NEURONS

In larval *X. laevis*, MCs characteristically have more than one primary dendrite that project into more than one glomerulus, mainly into two or three glomeruli. None of the investigated MCs projected into a single glomerulus (Nezlin and Schild 2005). Therefore, the MC dendrites bifurcate in a way similar to the OSN projections described above. There might be a connection between the glomerular projection patterns of MCs and ORNs. In adult *X. laevis*, it is not known whether MCs innervate multiple glomeruli. Contrary to what is known for adult mammals (Shipley and Ennis 1996; Lledo et al. 2005), in adult turtles, MCs stereotypically innervate two glomeruli (Mori et al. 1981). This shows that glomerular projections of MCs in adult lower vertebrates can differ from those in mammals.

In mammals, the prenatal morphogenesis of MC dendrites follows a precise scheme. Immature MCs first extend undifferentiated dendrites with radial orientation toward the developing glomerular layer where ORN axons start to coalesce, innervating several adjacent glomeruli. With ongoing maturation, all but one dendrite retract and only a single primary dendrite, forming a glomerular tuft, stays in the glomerular layer. Differentiated secondary dendrites appear first in postnatal animals. Within the first two postnatal weeks, the maturation of MCs terminates (Malun and Brunjes 1996; Matsutani and Yamamoto 2000). The number of glomeruli innervated by a single MC in adult *X. laevis* remains to be determined.

# 4.5 MODULATION AND SIGNALING IN THE OLFACTORY EPITHELIUM (OE)

The OE is usually considered as the site where individual ORNs, every ORN independently from all others, detect odorants. Most studies on olfactory transduction were done in dissociated ORNs (Schild and Restrepo 1998). The OB has thus been thought of as the first level for odorant information processing. Consequently, the importance of multicellular interactions in the OE and the impact of efferent innervation on odorant transduction received little attention. In recent years, the importance of modulatory events in the OE is becoming increasingly evident. The list of substances that have been shown to act as signaling molecules in the olfactory neuroepithelium and/or to have influence on peripheral odorant processing includes various neurotransmitters (Bouvet et al. 1988; Vargas and Lucero 1999; Hegg and Lucero 2004; Mousley et al. 2006), nucleotides (Hegg et al. 2003; Hassenklöver et al. 2008), endocannabinoids (Czesnik et al. 2007), and hormones (Arechiga and Alcocer 1969; Kawai et al. 1999; Eisthen et al. 2000). The modulation of olfactory sensory neurons by these substances match odorant sensitivity to the appetitive, arousal, reproductive, or injury state of the animal, thus impacting multiple physiological processes, including feeding behavior, mating, as well as local neuroprotective and regenerative processes.

## 4.5.1 PURINERGIC SYSTEM

It has been shown that cells in the vertebrate OE express purinergic receptors (mouse: Hegg et al. 2003, 2008; larval *X. laevis*: Czesnik et al. 2006; Hassenklöver et al. 2008). In larval *X. laevis*, the application of adenosine triphosphate (ATP) evokes strong increases in the  $[Ca^{2+}]_i$  in SCs (Hassenklöver et al. 2008). Specifically, the responses follow a characteristic spatiotemporal pattern. The onset of the  $[Ca^{2+}]_i$  increase always occurs in the apical part of the SCs and subsequently

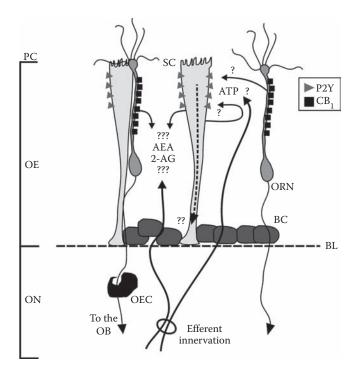
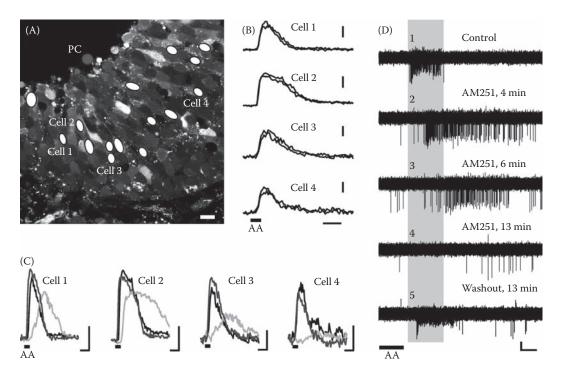


FIGURE 4.5 Schematic representation of intraepithelial signaling mechanisms and modulatory events in the olfactory epithelium of larval Xenopus laevis. Nucleotides, locally released from cells in the OE or from efferent nerve fibres terminating in the OE, induce strong increases in the [Ca<sup>2+</sup>]<sub>i</sub> via activation of purinergic receptors located on SCs. These responses follow a characteristic spatiotemporal pattern. The onset of the  $[Ca^{2+}]_{i}$  increase always occurs in the apical part of the SCs and subsequently propagates along their basal processes toward the basal lamina. This strongly suggests that the purinergic receptors may be localized on the soma of the SCs (see Hassenklöver, T. et al. Glia 56, 1614-24, 2008). This nucleotide-induced "calcium wave" could serve as an intraepithelial communication pathway from the very apical part of the OE to the basal lamina where BCs reside. CB<sub>1</sub>-like immunoreactivity could be localized on ORN dendrites indicating that CB<sub>1</sub> receptors are localized on ORN dendrites. Activation of CB<sub>1</sub> receptors modulates odor-evoked [Ca<sup>2+</sup>] changes and electrical activity (action potentials) of ORNs. This indicates that the epithelial endocannabinergic system has a profound impact on peripheral odor processing. At present, it is not known which endocannabinoids are responsible for this effect, or if the endocannabinoids are locally released from cells of the OE or from efferent nerve fibres terminating in the OE (see Czesnik, D., Schild, D., Kuduz, J., and Manzin, I., Proc Natl Acad Sci USA., 104, 2967–72, 2007). (PC, principal cavity; OE, olfactory epithelium; ON, olfactory nerve; SC, sustentacular cell; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; OEC, olfactory ensheathing cell; OB, olfactory bulb; BL, basal lamina; BC, basal cell; ORN, olfactory receptor neuron; ATP, adenosine triphosphate; P2Y, purinergic receptor; CB<sub>1</sub>, cannabinoid receptor type 1.)

propagates along their basal processes toward the basal lamina. This strongly suggests that the purinergic receptors may be localized on the soma of the SCs. A thorough pharmacological characterization of the purinergic responses suggests that extracellular nucleotides in the OE activate SCs via  $P2Y_2/P2Y_4$ -like receptors (Hassenklöver et al. 2008).

In other sensory systems, e.g., in the visual, auditory, and gustatory system, extracellular nucleotides have long been known to have neuromodulatory effects and to be involved in cellular signaling (Burnstock 2007; Thorne and Housley 1996). At present, we can only speculate about the physiological role of the characteristic purinergic signaling in SCs in the OE of larval *X. laevis*.

In the OE of mouse, SCs are reported to express solely P2Y receptors, whereas ORNs have been shown to express both P2X and P2Y receptors (Hegg et al. 2003). Coapplication of nucleotides and odorants suppressed odorant-induced  $[Ca^{2+}]_i$  increases of mouse ORNs. Therefore, it has been



**FIGURE 4.6** The CB1 antagonist, AM251, alters odor-evoked  $[Ca^{2+}]_i$  changes and electrical activity in individual ORNs of larval *Xenopus laevis*. A: Fluo4-AM-stained acute slice preparation of the OE (image acquired at rest; PC, principal cavity). The white ovals indicate the ORN somata of this slice that responded to a mixture of amino acids (scale bar 10 µm). B:  $[Ca^{2+}]_i$  transients of individual ORNs upon repeated applications of the amino acids are highly reproducible. The intraepithelial location of the four cells shown is indicated in A (scale bars: 10 s and  $\Delta$ F/F 100% [cells 1 and 2] and 10 s and  $\Delta$ F/F 50% [cells 3 and 4]). C: After the addition of AM251 to the bath solution, the amino acid-evoked ORN responses (black traces) were modulated (light gray traces). After 12 min of drug washout, the amino acid-induced  $[Ca^{2+}]_i$  transients recovered completely (dark gray traces) (scale bars: 10 s and  $\Delta$ F/F 100% [cells 1 and 2] and 10 s and  $\Delta$ F/F 50% [cells 3 and 4]). D: Amino acid-induced action potential-associated currents of an individual ORN (scale bars: 2 s and 20 pA [D1–D5]). The modulatory effect of AM251 on the action potential-associated currents depends on the wash-in time of the antagonist (A2–A4). Recovery after 20 min of drug washout (A5). The gray-shaded area indicates the time window of the original response. (Modified from Czesnik, D., Schild, D., Kuduz, J., and Manzin, I., *Proc. Natl. Acad. Sci. USA.*, 104, 2967–72, 2007.)

suggested that purinergic receptors in the mouse OE may play a role in signaling acute damage to the OE and that ATP release of damaged cells in the OE may prevent overstimulation of cells in the olfactory system during regeneration. Additionally, purines induce the expression of heat-shock proteins in SCs, which appears to initiate a form of neuroprotection in the OE (Hegg and Lucero 2006). In contrast, in the OE of larval *X. laevis*, ORNs do not appear to express purinergic receptors. We have shown that in larval *X. laevis*, only SCs but not ORNs are activated by extracellular nucleotides. This shows that there are differences in the purinergic systems in the OE of mouse and *X. laevis*. In contrast to what has been shown for mice, the purinergic system in the OE of larval *X. laevis* appears to serve as an intraepithelial communication pathway from the very apical part of the OE to the basal lamina (Figure 4.5).

## 4.5.2 ENDOCANNABINERGIC SYSTEM (ECS)

It has been shown that in the OE of larval *X. laevis*, cannabinergic substances have a profound impact on odorant-induced responses of ORNs (Figures 4.5 and 4.6; Czesnik et al. 2007). In ORNs,

specific CB<sub>1</sub> receptor antagonists, such as AM251, AM281, and LY320135, decrease the amplitude of odor-evoked  $[Ca^{2+}]_i$  responses and increase the latency of such signals. A comparable modulatory effect by AM251 was observed in patch clamp experiments. Spiking responses were increasingly delayed, and became longer and weaker. Consistently, application of a highly specific CB<sub>1</sub> agonist (HU210) drastically accelerated the recovery during washout and increased the percentage of recovering responses. CB<sub>1</sub>-like immunoreactivity could be localized to the dendrites of ORNs (Czesnik et al. 2007). ORN dendrites are certainly the appropriate compartment for partially decoupling the transduction compartment from the transformation compartment. The effects of cannabinoids on odor-evoked ORN responses described above, may be explained by the dendritic localization of CB<sub>1</sub> receptors. *X. laevis* is the first species where such an effect has been shown. The underlying physiological processes of the endocannabinergic system in the OE remain to be elucidated.

Recently, several studies have been published dealing with the influence of the nutritious status on the neurophysiology of olfactory information processing and vice versa, whereby some of the phenomena could indirectly be attributed to the effects of modulators like orexin in the rat OB or neuropeptide Y in the OE (Cailoll et al. 2003; Apfelbaum et al. 2005; Hardy et al. 2005; Mousley et al. 2006). The endocannabinergic system is known to be involved in food intake and energy homeostasis (Di Marzo and Matias 2005). Indeed, in several species, brain endocannabinoids seem to act as orexigenic mediators (Valenti et al. 2005; Soderstrom et al. 2004; Kirkham et al. 2002; Di Marzo et al. 2001). Our findings together with the abovementioned observations and the known role of olfaction in food detection certainly support the idea that the endocannabinergic system may play an important role in the response of organisms to their nutritional status. The main effect may be that the hungrier an animal is, the more sensitive are its ORNs. Similar effects may exist in other sensing systems.

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# 5 Development of the Olfactory System

Helen B. Treloar, Alexandra M. Miller, Arundhati Ray, and Charles A. Greer

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# 5.1 INTRODUCTION

Several excellent reviews have detailed the conservation of principles between insect, amphibian, and mammalian olfactory systems, and many important contributions to the field have been made by cross-phyla comparisons. However, within the limitations of this reference, in this chapter we will emphasize embryonic development and axon targeting in the rodent olfactory system, focusing on the mouse. To aide comparisons between studies in other rodents, the developmental ages in studies in the rat will be adjusted and referred to as the equivalent developmental age in mice. We apologize for any papers we may have missed or not had the opportunity to discuss in detail due to space limitations.

The olfactory system is one of the most precocious sensory systems to develop in the embryo. The primary olfactory pathway is comprised of two components, the olfactory epithelium (OE) and the olfactory bulb (OB). The secondary olfactory pathway includes multiple cortical regions,

all of which are directly innervated by OB projection neurons, and are collectively referred to as the olfactory cortex. Major regions of the olfactory cortex include the anterior olfactory nucleus (AON), the olfactory tubercle, the piriform cortex, and the entorhinal cortex (reviewed in Wilson et al. 2006). These regions are innervated by mitral and/or tufted cell axons via the lateral olfactory tract (LOT).

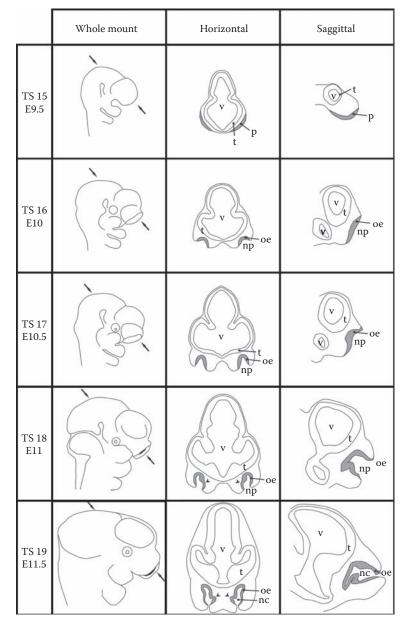
During the earliest stages of primary olfactory pathway formation, the OE and the OB undergo simultaneous, yet independent, developmental programs (López-Mascaraque and De Castro 2002). However, as development progresses and axons from the OE innervate the nascent OB, their developmental programs become interrelated (Treloar et al. 1999; Matsutani and Yamamoto 2000; López-Mascaraque et al. 2005). The early separation in developmental programs is perhaps not surprising given the spatial segregation of the regions that give rise to these two structures (see below). Despite a common ectodemal origin, the OE (the peripheral component of the olfactory system) is derived from the olfactory placode, while the OB emerges from the germinal zones of the neural tube, like other central nervous system (CNS) structures.

#### 5.2 FORMATION OF THE NASAL CAVITY

The olfactory placodes that give rise to the OE are specialized areas of cranial non-neural ectoderm found in the rostrolateral regions of the head. This specialized epithelial thickening invaginates to form a simple nasal pit. Like the majority of placodes, some mesenchymal cells migrate away from the placodal epithelium and differentiate as either secretory cells or glial cells (Scholsser et al. 2006). In rodents, the formation of the nasal pit occurs soon after the closure of the neural tube; the formation of the OE precedes the formation of the OB. In mice, the olfactory placodes are identified as epithelial thickenings as early as embryonic day 9 (E9), which is equivalent to Theiler Stage 14 (TS14; Theiler 1972). It must be noted that the embryonic days stated here are based on E0 being designated the day a positive vaginal plug is identified on the morning after mating. Due to differences in criteria between researchers in naming embryonic stages by days, we have included the Theiler stages as an unambiguous standard, which can easily be cross-referenced with common resources such as The House Mouse: Atlas of Mouse Development (Thieler 1972), The Atlas of Mouse Development (Kaufman 1992), and the Emap Digital Atlas of Mouse Development (http://genex.hgu.mrc.ac.uk/Atlas/intro.html). It is important for readers to note that careful attention should be paid to the staging and nomenclature strategy adopted by individual researchers, as this is crucial for interpretation and for comparison between studies. Staging embryos using established developmental landmarks is essential; at early stages, individual embryos can vary by as much as two to three stages in their development across the uterine horn, making staging by days postconception (dpc) insufficient for early developmental analyses.

By E10/TS16, the olfactory placode has thickened considerably since its initial appearance and begins to invaginate, appearing much like a simple bowl. This structure is referred to as the olfactory pit and is the beginning of the nasal cavity. Twelve hours later (E10.5/TS17), the nasal pits have invaginated further, forming distinct marginal rims. At E11/TS18, the olfactory pit has deepened considerably and the rims are beginning to unite, forming the nostrils. By E11.5/TS19, the nostrils are narrowed to small slits and the nasal pit has further invaginated into a more complex nasal cavity. In the medial wall of the newly formed nasal cavity, the vomeronasal organ (VNO) has invaginated further into a separate, distinct cavity. Thus, by E11.5/TS19, the main olfactory and vomeronasal cavities/epithelia can be distinguished. A schematic representation of the formation of the nasal cavity from the olfactory placode is shown in Figure 5.1.

During this period, there is a marked realignment of the polarity of the olfactory system, from rostrolateral to rostral. While the placodes are initially laterally located on the embryo, as development proceeds and the nasal pits and nostrils form, the nasal cavity shifts orientation from a



**FIGURE 5.1** Schematic diagram of the developing nasal cavity from Thieler Stage (TS) 15/embryonic day 9.5 (E9.5) to TS19/E11.5. At TS15, the olfactory placodes (shaded gray) are located very laterally, and are only a few cell layers thick. By TS16, the placodes have invaginated to form simple nasal pits. At TS17, the pits have deepened, but still maintain a simple cuplike morphology. At TS18, a second invagination in the medial wall of the nasal pit is evident (arrowheads). This second invagination is the initial formation of the vomeronasal organ. At TS19, the openings of the pits have constricted, forming the nares. The nasal cavity has deepened and become more complex. The vomeronasal pit has also deepened. As development proceeds, the nasal structures reorient rostrally from a more lateral position on the head. This is particularly evident in the horizontal view. The arrows in the whole mount diagrams indicate the plane of the horizontal diagrams. Abbreviations: t, telencephalon; v, ventricle; nc, nasal cavity; np, nasal pit; p, placode; oe, olfactory epithelium.

rostrolateral position to a more rostral location. This is best seen in horizontal sections through the head (Figure 5.1); the placodes are laterally located and as they invaginate and nasal pits form, the orientation of the developing nasal cavity gradually moves rostrally until the nares form at the most rostral tip of the head.

#### 5.2.1 MOLECULAR BASIS OF NASAL PIT FORMATION

The signals that control olfactory placodal induction are not as well understood as other sensory placodes. Perhaps the best insights into olfactory placode induction come from a recent study in chick, where several transcription factors were identified whose spatiotemporal expression patterns reflect olfactory fate acquisition (Bhattacharyya et al. 2008). Transcripts for Dlx5 and Pax6 are present quite early at the neurula stage, however four stages later, expression of Dlx5 is upregulated while expression of Pax6 is inversely downregulated. In contrast, Dlx3 is expressed at low levels early on, but is upregulated once cells are committed to an olfactory fate (Bhattacharyya et al. 2008). Furthermore, other work in *Xenopus* has demonstrated that the induction of the olfactory placode is blocked by hedgehog signaling (Cornesse et al. 2005). Recent studies in the mouse have identified the transcription factors *Sox2*, *Oct-1* (encoded by the *Pou2f1* gene), and *Pax6* as combinatorial components of the molecular pathway used to induce the olfactory placode (Donner et al. 2006). Mice with mutations in both *Sox2* and *Pou2f1* fail to undergo nasal morphogenesis, but mice with just a single mutation in either gene exhibit normal developmental staging (Donner et al. 2006). Similarly, in the *Pax6* mutant mouse small eye (*Pax6*<sup>Sey/Sey</sup>), the olfactory placodes fail to differentiate (Hill et al. 1991; Grindley et al. 1995).

Following placode induction, the subsequent development of the nasal cavity involves signaling by retinoic acid (RA), fibroblast growth factors (FGFs), and bone morphogenetic proteins (BMPs) from the adjacent frontonasal mesenchyme and olfactory ectoderm (e.g., LaMantia et al. 2000; Kawauchi et al. 2004, 2005; reviewed in Beites et al. 2005; Balmer and Lamantia 2005; Rawson and LaMantia 2006). RA was shown to be involved in mesenchymal/epithelial (M/E) signaling and, together with *Fgf8*, *Bmp4*, and Sonic hedgehog (*Shh*), is involved in defining axes in the developing OE and olfactory nerve (LaMantia et al. 2000; Bhasin et al. 2003; reviewed in Balmer and Lamantia 2005; Rawson and LaMantia 2006). Previous work has demonstrated that RA signaling between the placode and the associated mesenchyme is essential to generate both the molecular and cellular diversity of the OE and to establish the appropriate axon connections within the primary olfactory pathway (LaMantia et al. 2000).

Using in vitro assays, RA, Fgf8, Shh, and Bmp4 were found to provide different axial M/E signals: RA is a lateral signal, Bmp4 is a posterior signal, and Fgf8 and Shh are both medial signals. When their signaling is blocked or augmented in vitro, distinct aspects of olfactory pathway differentiation and patterning are compromised (LaMantia et al. 2000). Complementing these findings is a recent study on the role of Fgf8 in the developing olfactory system using a conditional knockout approach to create mice with Fgf8 inactivated in the OE from the earliest stages of development (Kawauchi et al. 2005). In normal development, Fgf8 mRNA is expressed in the rim of the invaginating nasal pit in a small domain of cells termed the morphogenetic center, which partially overlaps with the domain of putative OE neural stem cells later in gestation. In the Fgf8 null mice, the initial invagination of the nasal pit and the initiation of the OE differentiation occur, but development halts shortly thereafter due to apoptosis of cells in the morphogenetic center and adjacent developing neuroepithelium. Consequently, a definitive OE and VNO fail to develop in these mice. Thus, Fgf8 is crucial for the proper development of the OE, the nasal cavity, and the VNO (Kawauchi et al. 2005).

This process of molecular signaling between the mesenchyme and epithelium (M/E) is not unique to the olfactory system and is common at other sites of nonaxial induction, such as the limb buds, heart, and brachial arches (reviewed in Balmer and Lamantia 2005; Rawson and LaMantia 2006). At these sites (the olfactory placode included), local production of RA and the RA synthetic enzyme Raldh2 in the mesenchyme are autonomous (Bhasin et al. 2003). However, in the placode there are some differences in M/E signaling compared to the other sites, such as unique expression of RALDH3 in the placode (Bhasin et al. 2003; Kawauchi et al. 2004), and different effects of *Fgf8* on the RA receptor *RAR*b and *Raldh2* (Bhasin et al. 2003). These findings indicate that the olfactory system has apparently modified a general mechanism of M/E regulation to meet its requirements of establishing the primary olfactory pathway.

# 5.3 PRIMARY OLFACTORY EPITHELIAL GENESIS

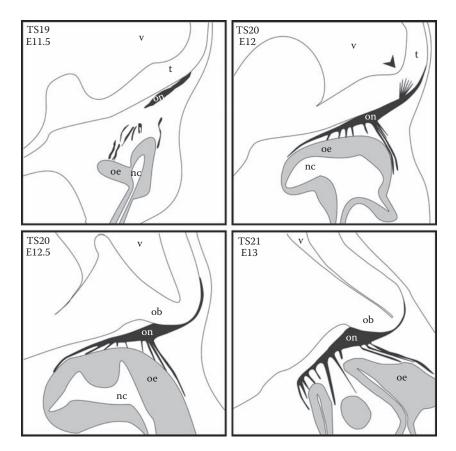
The OE is a pseudostratified neuroepithelium that comprises multiple cell types. In addition to mature and immature OSNs, which reside in the middle pseudolayer, there are two subpopulations of basal cells, the horizontal basal cells (HBCs) and the globose basal cells (GBCs), as well as supporting or sustentacular cells that reside in the apical pseudolayer. These cell types all differentiate after placodal induction and are thought to come from a common progenitor (reviewed in Nicolay et al. 2006). The lineage of olfactory sensory neurons (OSNs) has been established by a number of laboratories using a combination of in vitro and in vivo studies (reviewed in Kawauchi et al. 2004; Beites et al. 2005). Briefly, a population of self-renewing stem cells, probably the GBCs (Caggiano et al. 1994; Huard et al. 1998), give rise to a population of transit-amplifying cells that express mammalian achaete-scute homolog 1 (Mash1+), a basic helix-loop-helix (bHLH) transcription factor essential for OSN development (Guillemot et al. 1993). Mash1+ cells give rise to a second transit-amplifying progenitor, the intermediate precursor (INP), which expresses another bHLH transcription factor, neurogenin1 (Ngn1) (Cau et al. 1997, 2002). Daughter cells from dividing INPs differentiate into OSNs, which are easily identified by a variety of markers, including the neural cell adhesion molecule (NCAM) and the olfactory marker protein (OMP) (reviewed in Nicolay et al. 2006).

Interestingly, at the very earliest stages of epithelial development, neurogenesis follows a slightly different scheme than later in embryogenesis, postnatal and adult stages (see Chapter 10). At early embryonic stages (e.g., E11–E14), the majority of dividing cells are found apically in the developing OE, while later in development proliferation is found predominantly near the base of the OE (Smart 1971; Carson et al. 2006).

# 5.4 DEVELOPMENT OF THE OLFACTORY NERVE

Soon after OSNs differentiate, they extend an axon that pierces the basement membrane of the OE, enters the underlying frontonasal mesenchyme, and begins the process(es) of navigating from the developing OE to the rostral portion of the telencephalon where the OB will develop. OSN axons do not migrate independently, a population of migratory cells also exits the olfactory placode and migrates with the OSN axons, forming an accumulation of axons and cells that have been termed the "migratory mass" (Valverde et al. 1992). The exact nature of the migratory cells is still uncertain, but probably includes precursors of the olfactory ensheathing glial cells (Valverde et al. 1992), some cells that migrate to other brain regions, including the (gonadotropin-releasing hormone (GnRH) or LHRH) expressing cells (Schwanzel-Fukuda and Pfaff 1989; Wray et al. 1989a, 1989b; Valverde et al. 1993; De Carlos et al. 1995), as well as some migratory cells that are OMP+, express OR proteins and may be putative "guidepost" cells for growing OSN axons (Conzelmann et al. 2002). It is not yet established whether OSN axons or migratory cells exit the placode first, but both axons and cells exit very early in embryonic development and migrate through the mesenchyme medially, toward the telencephalon (Whitesides and LaMantia 1996). OSN axons exit the OE and make an immediate turn toward the telencephalon (Treloar et al. 1996; Whitesides and LaMantia 1996). To date, it is unclear which cues underlie this highly stereotypic turn, but differentially expressed ECM molecules in the frontonasal mesenchyme may form a permissive pathway for growing OSN axons (Whitesides and LaMantia 1996).

The formation of the olfactory pathway is unique among sensory systems in that OSNs are the only sensory transduction cells that do not follow established migratory pathways (reviewed in Balmer and LaMantia 2005). The initial establishment of the olfactory nerve involves OSN axons and migratory cells pioneering a pathway, using guidance cues present in the mesenchyme as well as chemotrophic cues released from the telencephalon. The migratory mass grows/projects along the mediorostral surface of the telencephalon, not coming in contact with its surface until E11.5 in mice, when it establishes contact with the rostral-most tip of the telencephalon (Figure 5.2). By E12, the migratory mass has formed a presumptive olfactory nerve layer (pONL; Figure 5.2), with OSN axons entering the CNS though small fenestrations in the basement membrane of the telencephalon (Marin-Padilla and Amieva 1989; Treloar et al. 1996; Gong and Shipley 1996; Balmer and LaMantia 2004; Figure 5.2). A small subpopulation of OSN axons do not remain restricted to the pONL, instead they extend into the ventricular zone of the telencephalon (Pellier et al. 1994; Figure 5.2), where they are hypothesized to stimulate OB formation (see below) (Gong and Shipley 1995). By



**FIGURE 5.2** Schematic diagram of olfactory nerve formation from TS19/E11.5 to TS21/E13. At TS19, OSN axons have first contacted the telencephalon, though they have not penetrated the basement membrane. Twelve hours later, at E12 (early TS20), OSN axons penetrate the basement membrane, forming an olfactory nerve layer in the presumptive olfactory bulb, and a small subset of OSN axons are observed to penetrate deeper into the telencephalon. At this early stage, the olfactory bulb is not macroscopically distinct, but in sagittal sections a flexure in the telencephalon is clearly observed (see arrowhead). At E12.5 (late TS20), the olfactory bulb becomes macroscopically distinct, as a clear evagination from the telencephalon. OSN axons form a distinct nerve that terminates in a nerve layer that cups the newly formed olfactory bulb. The deep penetrating OSN axons are no longer observed. At TS21/E13, the olfactory nerve has increased in size as more OSN axons have extended to the olfactory bulb. Abbreviations: t, telencephalon; v, ventricles; on, olfactory nerve; nc, nasal cavity; ob, olfactory bulb; oe, olfactory epithelium

E12.5, these deep penetrating axons are no longer detected in the ventricular zone, although the fate of these axons is unknown. OSN axons remain restricted to the pONL until glomerulogenesis begins (see below), around E15 in mice (Treloar et al. 1999). During this four-day window, from E11.5 to E15, from when the axons first contact the telencephalon and form a pONL to when the axons grow deeper into the OB and establish synaptic connections, a continued growth of OSN axons into the pONL occurs as more OSNs are generated. It has been hypothesized that this waiting period in the pONL is crucial for OSN axons to sort prior to forming appropriate topographic connections in the OB (see below) (Treloar et al. 1999).

#### 5.4.1 MOLECULAR BASIS OF OLFACTORY NERVE FORMATION

While relatively little is known about the molecular cues that underlie the earliest stages of olfactory nerve formation, studies from transgenic mice have provided insight into some of the molecules that are involved in this process. Perturbation in a number of transcription factors is associated with anomalies in the formation of the olfactory nerve. These include Fez, Klf7, Arx, Emx2, Gli3, Six1, Dlx5, and the hypomorphic Fgf8; all have resulted in a similar phenotype in the olfactory nerve pathway. In most of these transgenics, the OSNs differentiated normally and extended axons, but deficits were seen in the formation of the olfactory nerve (Yoshida et al. 1997; Mallamaci et al. 1998; Yoshihara et al. 2004; Balmer and LaMantia 2004; Laub et al. 2005; Merlo et al. 2007; Ikeda et al. 2007; Chung 2008). OSN axons defasciculate and project aberrantly near the forebrain, rarely entering the CNS. Despite the common phenotype in these knockouts, a common mechanism has not been identified. Furthermore, while many of these molecules are expressed in the OSNs (Dlx5, Six1, Fez, Emx2, Fgf8, Klf7), some are not (Arx, Gli3). This raises the question of whether the projection of OSN axons to the telencephalon is dependent on axon guidance mechanisms or if connectivity is controlled via non-cell-autonomous mechanisms. For example, these genes could affect the formation of the olfactory nerve pathway by affecting the migration and differentiation of migratory cells located along the olfactory nerve pathway, or by influencing axon-mesenchyme interactions via a cell nonautonomous mechanism (Merlo et al. 2007).

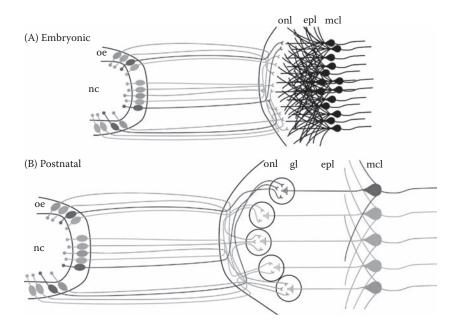
Also necessary for OSN axons targeting the OB is expression of a 7-transmembrane (7TM) receptor, like an odor receptor. Feinstein and Mombaerts (2004) demonstrate that when certain mutations are made in ORs, such as chimeric ORs or early stop codons, which they term neomorphic mutations, OSN axons expressing the mutated OR display poor outgrowth and fail to reach the OB. Thus, ORs appear necessary for OSN axon targeting to the OB. However, in mice that have had the beta-adrenergic 7TM receptor substituted into the M71 OR locus, the OSN axons can target or coalesce into glomeruli, indicating that 7TM receptor expression is not a unique axon guidance mechanism specific to ORs (Feinstein and Mombaerts 2004; cf. Chesler et al. 2007). Given the widespread expression of 7TM receptors within the nervous system, it is interesting to consider that 7TM receptor expression may be a ubiquitous axon guidance mechanism.

#### 5.5 DEVELOPMENT OF THE OLFACTORY BULB (OB)

The OB develops from a predetermined region of the rostral telencephalon (López-Mascaraque et al. 1996). During early embryonic development, the telencephalon is prespecified into different areas that develop into distinct adult brain regions. The initial model of area specification, first proposed by Rakic (1988), has received widespread support from numerous studies. The restricted expression patterns of transcription factors and signaling molecules underlie the patterning of the telencephalon (reviewed by Sur and Rubenstein 2005; Rash and Grove 2006; Mallamaci and Stoykova 2006). Graded cues have been identified that control the spatial formation of the axes of the developing telencephalon: FGF proteins set up the rostral–caudal (R–C) axis (Fukuchi-Shimogori and Grove 2001; Garel et al. 2003); Wnt and BMP proteins regulate dorsoventral (D-V) patterning (Furuta et al. 1997; Rubenstein et al. 1999; Hébert at al. 2002; Gunhaga et al. 2003); and medial-lateral

(M-L) patterning is thought to be regulated by the transcription factors *Emx2*, *Pax6*, and COUP-TF1 (Bishop et al. 2000; Mallamaci et al. 2000; Zhou et al. 2001).

FGF signaling has been implicated in OB specification (Meyers et al. 1998), but it is likely that other cues are also involved in the initial OB formation (Hébert et al. 2003). Macroscopically, the OB is first morphologically distinct at E12.5 as an evagination of the rostral telencephalon (Figure 5.3; Hinds 1968b; Sugisaki et al. 1996; Inaki et al. 2004), but, microscopically, changes can be observed in the rostral telencephalon prior to E12. After pioneer OSN axons have extended into the ventricular zone of the telencephalon (see above), a distinct flexure is observed in the rostral telencephalon (see Figure 5.3). Gong and Shipley (1995) examined the cell-cycle kinetics of cells in the presumptive OB (pOB) and the adjacent neocortex after the arrival of the pioneer axons. Twenty-four hours after the arrival of the axons, the duration of the cell cycle in the pOB was significantly prolonged compared to the cortex, suggesting that these axons influence the formation of the OB. However, the arrival of OSN axons postdates the differentiation of mitral cells, which begins on E10.5–11 (Hinds 1968a). Moreover, studies examining the Pax-6 mutant mouse Sey<sup>Neu</sup>/Sey<sup>Neu</sup> (Small eye), which lack an OE, revealed that an OB-like structure (OBLS) develops within the rostral telencephalon without inductive signals from OSN axons (Jiménez et al. 2000). Similarly, olfactory bulbs (OBs) develop in Dlx5deficient mice in which OSN axons extend from the OE, but fail to reach and innervate the OB (Long et al. 2003; Levi et al. 2003). These more recent studies suggest that OB formation occurs in the absence of any of the deep-penetrating OSN axons described by Gong and Shipley (1995). The exact nature of the signal that induces OB formation warrants further investigation (i.e., Shay et al. 2008).



**FIGURE 5.3** (See color insert following page 206.) Schematic diagram comparing embryonic (A) and postnatal (B) olfactory connectivity. During the postnatal period, OSNs express a single OR from a single allele (monoallelic expression, represented by different colors), and extend a single unbranched axon back to the olfactory bulb in large intermingled axon fascicles. OSN axons remain restricted to the ONL, directly apposed to a dense meshwork of dendrites of the developing projection neurons, the mitral and tufted cells. Mitral/tufted cell dendrites are very immature at this stage, with each neuron having multiple broadly spread apical dendrites. In the postnatal period, OSN axons have sorted out into "like-types" and targeted specific glomeruli. Mitral and tufted cells have undergone extensive remodeling to achieve their mature morphology of a single apical dendrite, which ramifies as an apical tuft within a glomerulus and numerous lateral dendrites that extend within the EPL. Abbreviations: nc, nasal cavity; oe, olfactory epithelium; mcl, mitral cell layer; epl, external plexiform layer; gl, glomerular layer; onl, olfactory nerve layer.

But, regardless of the molecules and mechanisms that underlie these processes, the olfactory system develops initially on two independent timeframes in the olfactory placode and the pOB; they become linked only as development continues (reviewed in López-Mascaraque and De Castro 2002).

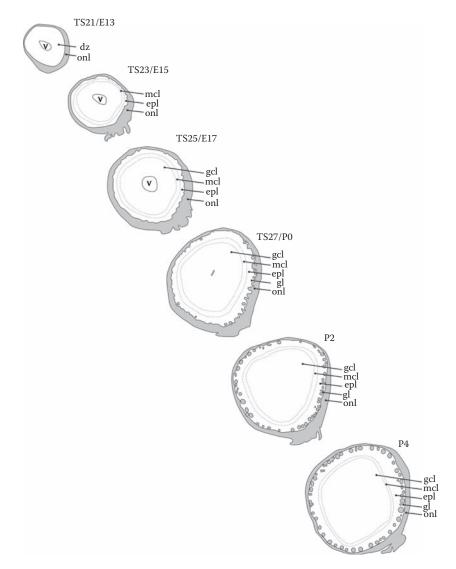
#### 5.5.1 **PROJECTION NEURONS**

Mitral/tufted (M/T) cell development has been categorized into three distinct phases: (1) a postneurogenesis phase (from E11 to E13), when newly generated cells migrate radially toward the pOB border and then undergo a tangential reorientation; (2) a sensitive period (from E14 to E16), during which cells orient radially upon arrival of OSN axons; and (3) a cell refinement phase (from E17 though adulthood), when cells extend their dendrites and undergo extensive pruning to acquire the mature morphology (Blanchart et al. 2006). During the sensitive period, developing M/T cells extend numerous dendrites apically, which form a presumptive external plexiform layer (pEPL), which is directly apposed to the pONL (Treloar at al. 1999; Bailey et al. 1999; Blanchart et al. 2006). The immature M/T cells have multiple broadly spread apical dendrites and they do not become associated with glomerularlike structures (protoglomeruli) until later in postnatal development. OSN axons remain restricted to the pONL until approximately E15, when they begin to grow in among the M/T dendrites in the pONL and begin to coalesce into protoglomerular structures at E17 (Treloar et al. 1999; Blanchart et al. 2006). M/T cell dendrites do not become associated with these protoglomerular structures until immediately prior to birth, when the uniform dendritic meshwork of the EPL begins to segregate into protoglomerular structures (Treloar et al. 1999).

M/T cells do not begin to refine their immature and broadly spread dendritic arbors (Figure 5.3A) into the mature morphology (Figure 5.3B) until the postnatal period. They undergo extensive pruning/remodeling until a single apical dendrite remains, which projects to and ramifies as an apical tuft within only one glomerulus and numerous lateral dendrites extend horizontally in the EPL (Malun and Brunjes 1996; Lin et al. 2000; Blanchart et al. 2006). This developmental process is believed to be at least partially dependent on the presence of neuronal activity and/or OSN axons (Lin et al. 2000; Matusnami and Yamamoto 2000). A recent report by Tran et al. (2008) examining mitral cell dendritic development in vitro suggests that TGF-beta, released by the OE, may influence dendritic growth.

Some morphologically distinct glomeruli can first be distinguished at birth, although glomerulogenesis appears to continue during the first postnatal days, while the exact timeline remains to be established (see Figure 5.4). Glomeruli do not form uniformly around the circumference of the OB, rather a gradient is seen with glomeruli forming closer to the points where OSNs enter the EPL and later in regions where OSNs take longer to enter the EPL (Blanchart et al. 2006). It will be interesting in future studies to compare the sites of early glomerulogenesis to the entry zones of OSN axons into the OB, as it seems likely that the first glomeruli to form will be from the earliest generated OSN axons and presumably the first axons to reach the OB.

Axogenesis of M/T cells starts shortly after final differentiation, around E11.5 (López-Mascaraque et al. 1996; Walz et al. 2006). Using mice with genetically labeled M/T cells, the first axons extend into the telencephalon at E11.5, and between E12 and E14 these axons elongate, forming an arch along the path of the future LOT (Walz et al. 2006). Prior to the extension of M/T axons, the position of the LOT can be identified by the presence of a subset of early generated neurons that are recognized by a monoclonal antibody (Lot1) (Sato et al. 1998). Lot1+ neurons appear necessary for LOT formation, as the LOT fails to form in organotypic cultures if the Lot1 cells are ablated. Between E15 and E16, the LOT extends fully, reaching the most caudal extent of the telencephalon, and the first axon collaterals are formed in the region of the AON and posterior piriform cortex (pPC). While the LOT appears formed by E16, from E17 until birth new axons are added to the LOT and growth of collaterals and innervation of higher cortical regions continues. By birth, most of the overall connectivity appears established, although refinement continues at least until the end of the second postnatal week (Walz et al. 2006).



**FIGURE 5.4** Schematic diagram of the developing olfactory bulb, detailing the emergence of glomeruli. At E13, OSN axons (illustrated in gray) reside in the ONL, directly apposed to a dense meshwork of dendrites from developing projections neurons. No intermingling of axons and dendrites occurs. At E15, the process of glomerulogenesis begins, with some OSN axons penetrating among the dendrites in the EPL. The MCL becomes morphologically apparent (see dashed lines). At E17, OSN axons begin to coalesce into glomerular-like structures, called protoglomeruli. Protogomeruli are not distinct from the ONL. By P0, some glomeruli are apparent, but many protoglomeruli are still present. At P2, many more glomeruli are observed and the MCL is thinner and more distinct. By P4, glomeruli are more numerous, and larger in size. Abbreviations: v, ventricle; gcl, granule cell layer; mcl, mitral cell layer; epl, external plexiform layer; gl, glomerular layer; onl, olfactory nerve layer; dz, dendritic zone.

## 5.5.2 INTERNEURONS

The two remaining largest populations of neurons within the OB are interneurons, the granule cells and periglomerular cells. These cells are largely generated during early postnatal life (Hinds 1968a; Altman 1969; Rosselli-Austin and Altman 1979; Bayer 1983). They are born in the subventricular zone lining the lateral ventricles and migrate into the OB along the rostral migratory stream (RMS)

(Luskin 1993). However, while most of the interneurons are generated between E18 and P5 (Hinds 1968a), recent studies have demonstrated that a pioneer population of OB interneurons are generated from precursors in the lateral ganglionic eminence (LGE) between E12.5 and E14.5, which migrate selectively into the pOB in a pathway that presages the RMS (Tucker et al. 2006). These early generated cells differentiate into a wide variety of mature interneurons and a significant number can still be detected 60 days postnatal, indicating that they are not a transient population. Tucker et al. (2006) point out that they probably underestimate the size of early generated interneurons because cells were only pulse-chased with BrdU once at E14.5; an examination of earlier time points will be necessary for understanding OB formation. How these early generated interneurons impact the formation of OB circuitry and synaptogenesis remains to be determined.

## 5.5.3 SYNAPTOGENESIS

The first synapses in the OB can be detected at E14, but are not found in appreciable numbers until E15 (Hind and Hind 1976; Hwang and Cohen 1985), which is coincident with OSN ingrowth into the pEPL and the initiation of glomerulogenesis (Treloar et al. 1999). Synaptogenesis in the three main neuropil regions of the OB (the glomeruli, the EPL, and the internal granule cell layer [GL]) are not uniform during prenatal development (Hinds and Hinds 1976). Synapse formation in the glomerular layer>EPL>internal GL. At E18, the most obvious differences are observed; synaptic density of the glomerular layer is higher than EPL, which is approximately tenfold that of the internal GL. By birth, virtually all synaptic types detected in adults have been found, but the number of synapses continues to increase. In the glomerular layer, the density of synapses reaches a peak around P15–P20, after which it slowly declines, while in the EPL and internal GL, synaptic density continues to increase slightly even up to P44, probably reflecting the addition of new interneurons (Whitman and Greer 2007a, 2007b).

# 5.5.4 MOLECULAR BASIS OF OLFACTORY BULB (OB) DEVELOPMENT

Much of the understanding of the molecular basis underlying the formation of the OB has come from studies of transgenic mice with defects in their OBs. As discussed above, many transcription and growth factors underlie specification of the rostral telencephalon and the pOB. Members of the fibroblast growth factor (FGF) family have been found to be important in patterning the telencephalon. In particular, several lines of evidence suggest that Fgf8 plays an important role in specifying the rostral-caudal axis of the rostral telencephalon (Crossley and Martin 1995; Shimamura and Rubenstein 1997; Meyers et al. 1998; Crossley et al. 2001; Fukuchi-Shimogori and Grove 2001). However, other FGFs, including Fgf15, Fgf17, and Fgf18, are also expressed in rostral telencephalon (McWhirter et al. 1997; Maruoka et al. 1998) and may play important roles in patterning this region. Although the FGF family is very large, with 22 genes identified in mice, they all mediate their responses through a family of four cell surface tyrosine kinase Fgf receptors (FGFRS; reviewed in Itoh and Ornitz 2004). Thus, approaching Fgf function during development is most feasible by targeting/deleting the receptor(s). In mice with a specific disruption of Fgfrl in the telencephalon (generated using Foxg1-Cre mice), OBs do not develop normally (Hébert et al. 2003). At E12.5, when the OB first becomes macroscopically distinct in wildtype animals (see above), through E16.5 OBs do not form in *Foxg1-Cre;Fgfr1<sup>flox/flox</sup>* mice. Between E18.5 and P0, a small OB protrusion develops, however it does not exhibit the characteristic lamination of wildtype OBs (Hébert et al. 2003). OSN axons have formed connections with this OB-like structure and the developing M/T cells have extended axons to higher cortical areas (see below). In wildtype animals, the reduced rate of proliferation in the rostral telencephalon (seen after the arrival of OSN axons) and the associated increase in differentiation relative to surrounding telencephalon (which continues to proliferate at a higher rate) is believed to trigger the evagination of the OB (Gong and Shipley 1995). In these telencephalon-specific Fgfrl null mice, no change in proliferation is observed in the rostral

telencephalon relative to the adjacent cortex after the arrival of OSN axons (Hébert et al. 2003), suggesting the Fgf signaling is playing a role in OB morphogenesis. Since an OB-like structure does form late in development, it appears that other Fgfrs are probably partially compensating for the loss of FGF signaling through Fgfr1.

#### 5.5.4.1 Projection Neurons

To date, only a few genes that are exclusively expressed in M/T cells have been identified. These include the transcription factors *Tbr1* (the mammalian brachyury homolog T-brain 1; Bulfone et al. 1995); *Tbx21*, another member of the *Tbr1* subfamily of T-box genes (Faedo et al. 2002; Yoshihara et al. 2005); *Id2*, a DNA-binding inhibitory helix-loop-helix Id protein (Neuman et al. 1993; Bulfone et al. 1998); neurotensin, a neurotransmitter transiently expressed by developing M/T cells (Kiyama et al. 1991; Walz et al. 2006), and *reelin*, a gene which encodes a secreted glycoprotein that was identified as having an autosomal mutation in the reeler trait (Schiffmann et al. 1997). Of these four genes, mice with null mutations have only indicated severe olfactory phenotypes with the *Tbr1* gene. Mice that lack *Tbr1* do form OBs, but they are small and do not have well defined layers (Bulfone et al. 1998). M/T cells fail to form, and the mice die within the first 2 days postnatal, as they do not suckle. Glomerular–like structures form within the mutant OBs, but this may reflect an inherent ability of OSN axons to coalesce rather than the formation of any synapses. Thus, *Tbr1* appears necessary for M/T cell, and ultimately OB, development.

#### 5.5.4.2 Interneurons

The interneuron populations also express many transcription factors, which when knocked out result in olfactory phenotypes. *Arx* is a vertebrate X-linked *prd*-type homeobox gene expressed by GABAergic interneurons in the OB (Poirier et al. 2004; Yoshihara et al. 2005). *Arx*-deficient mice die soon after birth and the neonatal mice have smaller OBs (Kitamura et al. 2002), primarily due to deficits in the entry of interneuron progenitors into the OB as well as disruptions in OB lamination (Yoshihara et al. 2005). Given the disorganized lamination and reduced size of the OB, as well as the neonatal lethality, *Arx* expression by interneuron precursors appears to be important for the development of the OB.

Another transcription factor, Sp8, which is a member of the Sp1 zinc finger gene family, is expressed by specific subpopulations of OB interneurons including the calretinin-expressing and GABAergic/TH-negative periglomerular cells (Waclaw et al. 2006). Sp8-deficient mice display severe exencephaly, making the analysis of OB formation difficult (Bell et al. 2003; Treichel et al. 2003). However, conditional mutations in Sp8 lead to a severe reduction in embryonic OB interneurons (Waclaw et al. 2006). During postnatal development, more interneurons reach the OB, however lamination defects are distinct, and deficits in specific subpopulations of interneurons are observed (i.e., they misexpress Pax6 and display abnormal migratory behavior) (Waclaw et al. 2006). Thus, Sp8 appears to play an important role in the regulation of interneuron development.

*Distal-less/Dlx* homeodomain transcription factors regulate the development of multiple cell types derived from the subcortical telencephalon, including the interneurons of the OB (Qiu et al. 1995; Anderson et al. 1997; Bulfone et al. 1998). *Dlx1*, *Dlx2*, *Dlx5*, and *Dlx6* are expressed in precursors of OB interneurons (Liu et al. 1997; Stuhmer et al. 2002), with *Dlx1* and *Dlx2* expression usually preceding that of *Dlx5* and *Dlx6* (reviewed in Pangnaiban and Rubenstein 2002). In mice deficient for either *Dlx1* or *Dlx2*, the population of TH-expressing periglomerular cells is reduced (Qiu et al. 1995; Long et al. 2003), but mice deficient for both *Dlx1* and *Dlx2* have a severe loss (>95%) of GABAergic interneurons (Anderson et al. 1997; Bulfone et al. 1998). As discussed above, mice deficient in *Dlx5* OSNs develop, but fail to form connections with the OB (Long et al. 2003; Levi et al. 2003). In addition to this peripheral phenotype, these mice also have a marked decrease in interneuron populations, particularly in the GAD65+/+, GAD67+/+, and TH+/+ populations of granule and periglomerular cells, as well as non-cell-autonomous effects in the mitral cell population, probably due to the absence of input from the OE (Long et al. 2003).

# 5.6 OLFACTORY SENSORY NEURON TARGETING OLFACTORY EPITHELIUM (OE)—BULB TOPOGRAPHY

Like all sensory systems, the axonal connections in the olfactory system, from the periphery to the CNS, are not random. But unlike other sensory systems, the strategy employed by the olfactory system is unique. In contrast to visual and somatosensory stimuli, olfactory stimuli are not spatially fixed, therefore a spatial representation of the sensory field in the CNS is not a prerequisite when coding olfactory information. Odors are volatile aromatics with distinct chemical properties, varying in their ability to diffuse through the air as well as the mucous bathing the OE, thus the relatively crude spatial patterning of ORs in the OE may reflect the chemical properties of the OR ligands.

#### 5.6.1 TOPOGRAPHIC ORGANIZATION OF OLFACTORY SENSORY NEURON PROJECTIONS

Each OSN expresses a single odorant receptor protein from a single allele (allelic exclusion) (Buck and Axel 1991; Chess et al. 1994; Malnic et al. 1999; Serizawa et al. 2000; see also Chapter 7). Negative feedback from the expressed OR molecules may maintain this one neuron-one receptor rule, although the mechanism remains unclear (Serizawa et al. 2003; Lewcock and Reed 2004; Shykind et al. 2004). Allelic exclusion is a key feature of olfactory biology, ensuring that each neuron is receptive to a unique, defined repertoire of ligands. OSNs expressing the same OR genes are expressed in restricted longitudinal bands of OE that vary in their dorsoventral position (Vassar et al. 1993; Ressler et al. 1993; Strotmann et al. 1994; Iwema et al. 2004; Miyamichi et al. 2005). The initial studies describing these restricted zones of expression identified only four zones (Vassar et al. 1993; Ressler et al. 1993; Strotmann et al. 1994). However, more recent studies recognize that rather than zones with distinct boundaries, there are instead continuous and overlapping expression domains that are unique for each OR (Iwema et al. 2004; Miyamichi et al. 2005).

Initially using a distinct property of OSNs (they contain mRNA in their axons) and later using molecular genetic tracing techniques, the axonal projection patterns of OSNs expressing the same OR were traced to their synaptic target glomeruli in the OB (Vassar et al. 1994; Ressler et al. 1994; Mombaerts et al. 1996; Wang et al. 1998). OSNs expressing the same OR converge on a few glomeruli in the OB, typically a pair, with one glomerulus on the medial surface and one on the lateral surface of each OB. This mosaic topographic pattern of divergent sensory neurons in the OE projecting and converging their axons onto a pair, or small number of glomeruli in the OB has been observed for all ORs examined to date. It is quite different from the point-to-point maps seen in the visual and somatosensory systems that maintain the relationships between sensory neurons in the receptive field in the target fields. The topographic map in the olfactory pathway is also not invariant; while identified glomeruli (i.e., containing axons expressing the same OR) maintain the same general locale between animals, the "neighbor" relationships between glomeruli can vary by a few glomerular diameters and can show variability between OBs within the same animal (Strotmann et al. 2000; Serizawa et al. 2006). This variability is not yet well understood, but may reflect developmental mechanisms underlying the formation of glomeruli. The zonal, or regional, organization seen in the OE appears to be maintained in the OB (Saucier and Astic 1986; Ressler et al. 1994; Vasser et al. 1994; Schoenfeld et al. 1994; reviewed in Mori et al. 1999). OSNs located in the dorsal nasal cavity converge and form glomeruli in the dorsal OB, and ventrally located OSNs project their axons to the ventral OB. Thus, each of the four broad zones/regions is represented in the OB. It should be noted, however, that as it becomes recognized that distinct zonal boundaries do not exist in the OE, and instead there are gradients of each OR along the dorsomedial/ventrolateral axes of the OE, similar gradients are observed in the OB, recapitulating the patterns seen in the OE (Miyamichi et al. 2005).

The topography described here, of glomerular convergence and zonal projections, underlies coding in the olfactory system. As all OSN axons expressing the same OR receptor converge

on a stereotypic pair of glomeruli within the OB, decoding sensory input becomes a problem of recognizing which patterns of glomeruli have been activated by specific stimuli. Moreover, through a process of lateral inhibition, the system is extremely sensitive: the convergence of axons on a small number of projection neurons amplifies the signal, while lateral inhibition of surrounding projection neurons via local interneurons further amplifies the signal to noise ratio (reviewed in Mori et al. 1999). Thus, the olfactory system is capable of detecting and discriminating odorants in the partsper-billion range.

#### 5.6.2 DEVELOPMENT OF AN OLFACTORY TOPOGRAPHIC MAP

How does this mosaic topography develop? How do all the OSN axons expressing a single OR from a family of over 1200 genes, converge and form glomeruli in stereotypic positions? Perhaps the easiest model to imagine is that synaptic connections form between the OE and the OB, and OR gene choice occurs later, after connections have been made. This is an attractive model because the precision of targeting would not be active, but rather a retrograde event that could be imposed on the OE by the OB. However, onset of OR expression has been reported as early as E11.25 in mice (Conzelmann et al. 2001), which predates synaptogenesis by 3–4 days (Hinds and Hinds 1976). Even at the earliest embryonic ages, OSNs expressing the same OR have restricted zonal/regional expression patterns, suggesting that retrograde signals from the OB do not influence OR gene choice in the OE.

The question then becomes how can the identity of the OR expressed by OSNs be encoded or represented at growth cones during the formation of the olfactory pathway? Perhaps the most obvious way is by using the OR protein itself. For OR proteins to play an active role in the targeting of OSN axons, they also need to be expressed on axons and growth cones (in addition to their more classical site of expression in olfactory cilia). In transgenic mice that have had the coding region of an OR replaced with a reporter gene, OSN axons fail to converge and form glomeruli, instead they appear to wander within the ONL (Mombaerts et al. 1996; Wang et al. 1998; Feinstein and Mombaerts 2004, Feinstein et al. 2004). Perhaps more convincing evidence of the OR receptor having a role in targeting OSN axons comes from mice that have had the coding region of one OR substituted with the coding region of another OR (Mombaerts et al. 1996; Wang et al. 1998). In these mice, OSN axons change their targeting to a glomerulus in an intermediate position, between that of the host and donor glomeruli. Antibody localization studies have demonstrated expression of OR proteins in OSN axons (Barnea et al. 2004; Strotmann et al. 2004), as has a transgenic line of mice with an OR protein directly fused to a GFP reporter (Feinstein and Mombaerts 2004).

Consistent with the observation that OR proteins are determinants in targeting, even single amino acid changes in key residues of OR protein can alter the targeting of OSN axons, causing them to converge in intermediate or new glomeruli (Feinstein and Mombaerts 2004). These studies collectively demonstrate the requirement of the OR protein in OSN axon targeting. Yet, they also demonstrate that ORs alone are not sufficient for correct targeting. If they were sufficient, the receptor substitution experiments would have revealed complete switching of glomerular position from host to donor instead of the intermediate positions observed. Thus, it seems likely that other guidance cues and mechanisms must be acting in the olfactory pathway, together with the OR proteins, to determine the final points of axon convergence.

A generalized scheme of the development of sensory maps involves sequential activityindependent and activity-dependent mechanisms: axon guidance molecules are utilized to generate the course pattern of innervation of targets, while subsequent refinement of projections is achieved through activity-dependent processes (reviewed in Goodman and Shatz 1993; Katz and Shatz 1996; Tessier Lavigne and Goodman 1996). Not surprisingly, both activityindependent and activity-dependent mechanisms appear to be acting in the formation of the topographic olfactory projection.

#### 5.6.1.1 Role of Molecular Guidance Cues in the Development of a Topographic Map

While OR genes have been identified as the primary candidates for mediating OSN axon targeting, other guidance cues have been identified in establishing the topography in this pathway. Within the developing nervous system, major families of axon guidance cues have been identified, including the netrins, slits, semaphorins, and ephrins (reviewed in Dickson 2002). While these are not the only axon guidance cues, they are the best understood, and members of each of these families have been identified in the olfactory system.

Semaphorins are a family of secreted and transmembrane proteins that have been identified as axon guidance cues in many regions of the developing nervous system (reviewed in Fiore and Puschel 2003; Yazdani and Terman 2006). In mice lacking Sema3A, OSN axons expressing the Sema3A receptor neuropilin 1 (npn1) fail to target glomeruli in the lateral and medial OB like their wild-type (WT) counterparts, instead aberrantly targeting glomeruli in the rostral and ventral OB (Taniguchi et al. 2003; Schwarting et al. 2004). However, differences were observed between the two lines of null mice reported. In one line, OSN axons expressing the OR P2 failed to target their stereotypic lateral and medial glomeruli, instead targeting multiple glomeruli in the ventral OB (Schwarting et al. 2004). While in the other line, P2-expressing OSN axons targeted appropriately (Taniguchi et al. 2003). These mice did differ in their genetic background, which may account for some of the variation observed. While these differences require further investigation, both studies agreed that zonal/regional projections were disrupted based on the expression of cell surface markers. Examination of the glomerular activity patterns in Sema3A null mice via intrinsic optical imaging also revealed distorted glomerular maps (Taniguchi et al. 2003). Thus, the loss of the inhibitory Sema3A signal during development appears to disrupt the zonal/regional specification of the OB.

Another large family of axon guidance cues implicated in the formation of topographic maps in other sensory systems is the Eph receptor tyrosine kinases and their ligands, the ephrins (reviewed in Wilkinson 2001). During development, OSN axons transiently express members of the ephrin family, while cognate Eph receptors are expressed by target cells in the OB (Zhang et al. 1996, 1997; St. John et al. 2000, 2002; St. John and Key 2001; Cutforth et al. 2003). However, expression patterns are not like the gradients found in the retinotectal system. Rather, there is a mosaic-type pattern of expression with neighboring glomeruli expressing very different levels of the ligands (Cutforth et al. 2003). Although the ligands and receptors exhibit highly regulated spatiotemporal patterns of expression in both OSN axons and bulbar targets (St. John et al. 2002), the promiscuous nature of the ligands, which bind to multiple receptors, make it difficult to predict the interactions that may occur. To date, only one study has looked at the functional role that ephrins and Ephs play in establishing olfactory topography. In mice lacking ephrin-A3 and ephrin-A5, OSN axons expressing the ORs P2 and SR1 have glomeruli that are shifted caudally (Cutforth et al. 2003). In mice that overexpress ephrin-A5 only in the P2-expressing OSN axons, glomeruli are shifted rostral relative to wildtype P2 glomeruli (Cutforth et al. 2003). However, it has been suggested that the genetic techniques used in generating these mice (tricistronic constructs) may reduce levels of the P2 protein, which could have effects on targeting independent of the coexpressed protein (Mombaerts 2006). Thus, while it appears that Ephs and ephrins have a role in establishing the olfactory pathway, further studies are needed to fully elucidate the nature of that role.

Recently, insulin–like growth factors (IGF) have been implicated in mediolateral OB targeting (Scolnick et al. 2008). The IGF family members (IGF1 and IGF2) initiate signaling by activating their receptor IGF1R. During early development (E14.5), IGF1 is expressed in a gradient manner along the mediolateral axis (Scolnick et al. 2008). Expression is restricted to the mitral cell layer and glomerular layers by E18.5. IGF2 is expressed around the OB; IGF1R is expressed in OSNs and in axon fascicles. IGF mutagenesis causes axon mistargeting. Instead of innervating the lateral OB, sensory axons innervate ectopic ventromedial glomeruli. This suggests that IGF signaling may play a role in establishing the topography of the olfactory system.

The expression of cell surface sugars of proteoglycans, glycolipids, and glycoproteins has been proposed to provide a "sugar code" or "glycode" for growing axons (St. John et al. 2002; Holt and Dickson 2005). In the olfactory system, a large body of literature describes a number of lectins (carbohydrate-binding proteins) that bind subsets of OSNs in distinct patterns (reviewed in Plendl and Sinowatz 1998). The diversity in the expression of cell surface sugars has been proposed to underlie the development of the olfactory pathway. Most of these studies, however, do not identify which proteoglycan, protein, or lipid the carbohydrate moiety is attached to, making functional studies difficult. Two approaches that have been used to look at the functional relevance of sugars in the developing olfactory system are: (1) assess the role of endogenous lectins; and (2) assess the role of various glycosytransferases, synthetic enzymes in glycan production.

Galectin-1 is an endogenous lactose-binding lectin that has established roles in cell-cell and cellmatrix interactions (reviewed in Camby et al. 2006). In the olfactory system, galectin-1 is expressed by OSNs, ensheathing cells and M/T cells (Mahanthappa et al. 1994; Puche and Key 1996). In vitro, galectin-1 is a potent promoter of neurite outgrowth *i* (Puche et al. 1996). In mice lacking galectin-1, a subset of OSN axons that can be labeled with the plant lectin *Dolichos biflorus* agglutinin (DBA) fail to project to their correct targets in the dorsocaudal OB (Puche et al. 1996). Thus, galectin-1mediated carbohydrate interactions appear to play a role in pathfinding during development of the olfactory projection.

The glycosyltransferase,  $\beta$ 1,3-N-acetylglucosaminyltransferase 1 ( $\beta$ 3-GnT1), is a key enzyme in lactosamine glycan synthesis and is expressed by a subset of OSNs (Henion et al. 2005). In mice lacking  $\beta$ 3-GnT1, OB innervation and glomerular formation is perturbed in neonatal mice; OSN axons expressing the P2, I7, and M72 ORs fail to form glomeruli (Henion et al. 2005). By two weeks postnatal, lactosamine is re-expressed in these mice via a secondary pathway and a regrowth of axons into the glomerular layer occurs. Thus, the carbohydrate lactosamine also appears to have an important role in the formation of the olfactory projection.

Another glycosyltransferase, alpha(1,2)fucosyltransferase (FUT1), synthesizes the blood group H (BGH) carbohydrate alphaFuc(1,2)Gal, which is expressed by all mouse OSNs (e.g., Lipscomb et al. 2002). BGH is the acceptor substrate for a glycosyltransferase that synthesizes blood group A (BGA), which is expressed by a subset of vomeronasal sensory neurons (St. John et al. 2006). In mice that lack FUT1, a delay in the development of the ONL and glomerular layer is observed, but no deficits were seen on OSN targeting (St. John et al. 2006). However, when blood group A transferase (BGAT) was overexpressed on all OSNs using the OMP promoter, VNO axons overshot their targets in the accessory OB and OSNs were observed to make targeting errors (St. John et al. 2006). These studies lend further support to the notion that cell surface carbohydrates are important determinants of OSN axon extension, coalescence, and targeting.

Various cell adhesion molecules (CAMs) have also been proposed to be involved in establishing the topographic olfactory pathway, although the evidence for their involvement is less clear. OCAM, the olfactory CAM, is expressed in a zonal topographic pattern highly suggestive of a role in establishing the topographic projections (Yoshihara et al. 1997; Treloar et al. 2003). However, no disruptions in topography are observed in OCAM null mice (Walz et al. 2006). All OSNs, both immature and mature, express the NCAM (Terkelsen et al. 1989). Mice that lack the NCAM-180 isoform have delays in formation of the olfactory pathway, with OSN axons taking longer to target and form glomeruli, with many axons failing to exit the ONL (Treloar et al. 1997). However, OR-expressing OSN axons have not been assessed in these mice, which would aide the characterization of the delayed phenotype.

Recent evidence also implicates members of the Wnt/Fz family in the extension and perhaps coalesence/targeting of OSN axons (Rodriguez-Gil and Greer 2008). Members of the Wnt family are expressed along the olfactory pathway, the OSN axons express several Fz receptors, and moreover, Wnt5a induces accelerated growth of OSN axons in vitro. While further work remains to be done, the evidence thus far is provocative in suggesting a role for this family of axon guidance molecules in the olfactory system.

#### 5.6.1.2 Role of Functional Activity in the Development of a Topographic Map

When assessing the role of functional activity in the olfactory system, different components of activity must be considered. Activity includes both odor-evoked responses as well as spontaneous responses. While there has not been a great deal of investigation, several key studies have looked at the effects of activity-dependent mechanisms of topographic map formation. One strategy for assessing the role of odor-evoked activity is to block odor stimulation by surgically closing a naris at birth. Although much of the coalesence of OSN axons has occurred by birth, naris closure can still have a profound effect on the specificity of glomeruli. For example, Zou et al. (2004) found that during initial development, OSN axons expressing the same OR can coalesce in supernumerary glomeruli, some of which are heterogeneous for OR expression. Within a few postnatal days, the hypertrophy is corrected and the number of glomeruli stabilizes at approximately two per. However, in mice with unilateral naris closure, the development refinement, the loss of the supernumerary glomeruli, was significantly retarded and many glomeruli received heterogeneous axonal input from OSNs expressing different ORs. Thus, naris closure suggests that beyond any role in primary axon coalesence/targeting, functional activity is important for refining the specificity of glomerular innervation. Genetic approaches have proven to be more tractable to looking at the role of activity in topographic map formation.

Alternative strategies for addressing the role of functional activity in the development of the primary olfactory pathway have used genetic approaches to delete, downregulate, or upregulate downstream members of the OR transduction cascade. Among the first were mice lacking the alpha subunit of the olfactory cyclic nucleotide-gated channel, OCNC1 (now called CNGA2). The mice are anosmic (Brunet et al. 1996) and although the olfactory pathway appears largely intact, the effects of the loss of odor-evoked activity on axonal wiring are somewhat controversial (Lin et al. 2000; Zheng et al. 2000). In a conventional null mutation of OCNC1, axons expressing the P2 odor receptor converge appropriately, while those expressing the M72 odor receptor do not (Lin et al. 2000; Zheng et al. 2000). However, when OCNC1 is selectively mutated in some M72-expressing OSNs, using a "monoallelic deprivation" paradigm, M72 axons expressing OCNC1 segregate into distinct glomeruli from those not expressing OCNC1 (Zheng et al. 2000). These data suggest that postnatal odor-evoked activity does play a role in OSN axon targeting or coalesence, at least for some subsets of OSNs, but it also raises the question of when the odor-induced activity occurs. While there is evidence for in utero functional activity in the olfactory system (i.e., Pedersen et al. 1983), there are also data showing that the temporal onset of OR expression varies significantly among ORs and that some, perhaps, may not appear until perinatal periods (Sullivan et al. 1995; Greer lab unpublished observations). Also, there has been some question regarding the retention of odor transduction among subpopulations of OSNs in the CNGA2 mice (Lin et al. 2004), as well as the possibility that downstream signaling from the G-protein-coupled receptor could influence axons independent of functional CNG channels (Chesler et al. 2007).

Imai et al. (2006) and Chesler et al. (2007) have shown the importance of cAMP signaling in OSN axon extension/coalescence. In both cases, increased levels of cAMP led to the coalescence of OSN axons, independent of odor-induced activity via an OR. Although additional mechanisms may also be involved, Serizawa et al. (2006) further suggested that activity-dependent regulation of cell surface adhesion molecules, such as Kirrel 2 and Kirrel 3, perhaps mediated via cAMP, may contribute to the regulation OSN axon adhesion and coalescence into glomeruli. This suggestion is further supported by data showing that in AC3 knockout mice, OSN axon behavior is aberrant and that glomerulogenesis is perturbed (Zou et al. 2007). Kirrel2/Kirrel3 and their counterparts, ephrin-A5/EphA5, are expressed in a correlated manner with subsets of OR-expressing OSN axons. Therefore, Serizawa et al. (2006) proposed that axon targeting is achieved by expression of a combination of recognition/guidance cues whose expression levels are determined by activity. Coined the "neural identify code," it will be interesting to see if OR-correlated activity-dependent expression of axon guidance cues does mediate OSN axon fasciculation during development.

Spontaneous, odor-independent activity may also influence OSN axons. Yu et al. (2004) conditionally expressed the tetanus toxin light chain in OSNs, inhibiting synaptic vesicle release

and blocking both spontaneous and odor-evoked activity. However, blocking synaptic function did not have a significant effect on the development or topography of the primary olfactory pathway. However, when OSN activity was blocked by the overexpression of the inward rectifying K+ channel, Kir2.1, a delay in the ingrowth of OSN axons into the OB resulted, as well as a gross disorganization of dorsal glomeruli and targeting errors of subpopulations of OSN axons. Thus, while synaptic activity may not be a prerequisite for growth, coalescence, and sorting of OSN axons, electrical transients are required.

#### 5.7 SUMMARY

In this chapter, we have reviewed the basic principles of development and differentiation in the primary olfactory pathway. In particular, we focused on the mechanisms influencing the emergence of the OE and the innervation of the OB by OSN axons. The story is clearly complicated and tight spatiotemporal regulation of molecular expression is required in order for the pathway to develop correctly. Many challenges remain. While myriad molecular mechanisms have been identified in the placode, the developing olfactory pathway, and the OB, how these are integrated to form the highly complex topography between the OE and OB remains to be determined.

#### ACKNOWLEDGMENTS

The authors express their appreciation to members of their laboratories for helpful discussions and critical readings. Work in the laboratories of the authors has been generously supported by NIH-NIDCD and NIH-NIA.

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# 6 Pheromones and Mammalian Behavior

Peter A. Brennan

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# 6.1 INTRODUCTION

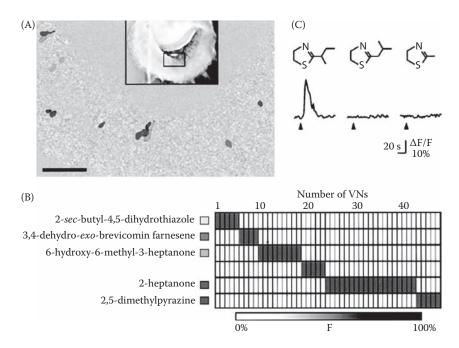
From the most gregarious to the most solitary, all animals have to coordinate their activity with other members of their species if they are to survive and reproduce. This requires some form of communication, which for the majority of animals involves the use of chemical signals, known as pheromones. Karlson and Lüscher (1959) initially proposed the term pheromone. They defined pheromones as "substances secreted to the outside of an individual and received by a second individual of the same species in which they release a specific reaction, for example, a definite behavior or developmental

process." Although not part of the original definition, the term pheromone is usually reserved for chemical signals that are produced and received by members of the same species, in which both the sender and receiver of the signal gain benefit (Wyatt 2003). In this case, selective pressures usually lead to the coevolution specialized sending and receiving systems for pheromones.

The identification of pheromones started in the 1950s with the purification of only 5.3 mg of the male silk moth attractant bombykol, from the scent glands of 313,000 female silk moths (Butenandt et al. 1959). Bombykol has since become a classic example of a sex attractant pheromone, attracting male silk moths over large distances. However, there has been considerable debate regarding whether the term pheromone, which was initially applied to insect chemosignals, can be usefully applied to vertebrates (Doty 2003). The issue comes down to what is meant by a "definite response." Vertebrate, especially mammalian, behavior is generally more dependent on context and learning than insect behavior, and therefore, responses to chemical signals are more difficult to observe, and rarely consistently effective in all individuals all of the time. This chapter reviews the recent evidence that has accumulated in support of mammalian pheromones that exert significant influence over mammalian physiology and behavior. In doing so, it takes a relatively broad view in discussing all intraspecific, specialized semiochemical signals as potential pheromones, while acknowledging that they may not meet the narrower interpretations of some researchers in the field.

#### 6.2 THE CHEMICAL NATURE OF PHEROMONES

A wide variety of chemicals are used as pheromones, including small, volatile molecules, proteins, and peptides (Figure 6.1), in which their chemical nature is linked to their function. Important



**FIGURE 6.1** (See color insert following page 206.) Stimulus selectivity of mouse vomeronasal class 1 (V1R)expressing vomeronasal sensory neurons (VSNs) recorded by Ca<sup>2+</sup> imaging from slices of the vomeronasal epithelium. (A) VSNs that responded to volatile pheromones were located in the apical region of the vomeronasal epithelium (area of image is shown by the box in the inset). Different response specificities are shown in different colors. (B) VSNs responded highly selectively to a single urinary volatile with known pheromonal activity. (C) A VSN that responded to 2*-sec*-butyl-4,5-dihydrothiazole failed to respond to stimuli with similar chemical features. (Reprinted by permission from Macmillan Publishers Ltd: [Nature] Leinders-Zufall, T. et al. *Nature*, 405, 792–96, copyright, 2000.)

features of chemicals used as pheromonal signals are their size and polarity, which determine their volatility in air and solubility in water. In the terrestrial environment, airborne signals that are required to act at a distance from the producer, such as attractant and alarm pheromones, need to be small and volatile, such as the male mouse urinary constituent, (methylthio)methanethiol (MTMT), which attracts female investigation (Lin et al. 2005). Their small size and volatility not only ensures that such pheromones are dispersed rapidly, but also makes these signals transient. In contrast, pheromonal signals that need to be associated with a specific individual or place in the environment are ideally nonvolatile, so that they do not disperse and are longer lasting. For example, male mice deposit urine marks containing 18–20 kDa major urinary proteins (MUPs), the stability and involatility of which make them ideal for their territorial marking role (Hurst and Beynon 2004).

# 6.3 PHEROMONE PRODUCTION

Animals use an enormous variety of different mechanisms for releasing pheromones into the environment (Table 6.1). In many cases, pheromonal release takes advantage of existing routes for excretion, such as urine and feces, which may be deliberately placed in the environment as territorial marks. For instance, the urine marks used by rodents, such as mice, are known to contain a variety of small, volatile pheromones (Novotny 2003), as well as sulfated steroids and proteins

## TABLE 6.1

# The Chemical Nature, Source, and Pheromonal Effects of a Range of Commonly Accepted Mammalian Pheromones

Chemosignal	Species	Sex	Туре	Secretion	Receptors	Effect
Androstenol	Pig	Male	Steroid	Saliva	MOE?	Female attraction+lordosis
4,16-Androstadien-3-one (AND)	Human	Male	Steroid	Axillary sweat	MOE?	Increased female cortisol
2-Methylbut-2-enal	Rabbit	Female	Volatile	Nipples/ milk	MOE?	Pup attraction+nipple search
(Methylthio)methanethiol (MTMT)	Mouse	Male	Volatile	Urine	MOE (TRPM5)	Female attraction
(R,R)-3,4-dehydro-exo- brevicomin (DB)+ (S)-2-sec-butyl-4,5- dihydrothiazole (SBT)	Mouse	Male	Volatile	Urine	VNO (V1R) MOE?	Male aggression Female estrus induction
Exocrine gland-secreting peptide (ESP) 1	Mouse	Male	Peptide	Tear secretions	VNO (V2R)	?
Major histocompatibility complex (MHC) peptides	Mouse	Both?	Peptide	?	VNO (V2R)	Mate recognition?
Major urinary proteins (MUPs)	Mouse	Male> female	Lipocalin protein	Urine	VNO (V2R)	Male counter marking Male aggression Female mate choice
Aphrodisin	Hamster	Female	Lipocalin protein	Vaginal fluid	VNO (V2R?)	Male sexual behavior

*Note:* MOE, main olfactory epithelium; TRPM5, transient receptor potential channel M5; VNO, vomeronasal organ; V1R, vomeronasal receptor class 1; V2R, vomeronasal receptor class 2.

that are also likely to have a pheromonal function (Chamero et al. 2007; Nodari et al. 2008). Other routes of pheromone release involve biological secretions. Hamsters release the sexual attractant protein, aphrodisin, in their vaginal secretions (Mägert et al. 1999). The rabbit mammary pheromone is produced by glands around the nipples and is present in rabbit milk (Schaal et al. 2003). Several potential chemosignals have been identified in the saliva of different species, including the well-known sexual attractant pheromone of boars (Loebel et al. 2000). But, there are also a wide variety of specialized scent glands that have no known role other than the release of pheromonal signals, even if, in most cases, little is known of the nature of the signals or the role that they perform. For instance, flank glands in hamsters can be used to leave marks that convey information about individual identity (Mateo and Johnston 2000). Most species of carnivora have anal glands, including ferrets, which produce sex-specific volatiles that could function as pheromones (Zhang et al. 2005). Other specialized scent glands include chin glands, interdigital glands, and sternal glands.

In addition to the analytical chemistry used for the analysis of volatile components of glandular secretions, modern molecular biological approaches are revealing a wide variety of proteins and peptides that are likely candidates for pheromonal signaling. A family of peptides, called exocrine gland secreting peptides (ESPs), has recently been identified in mice. The starting point for Touhara's group was the realization that chemicals released from the facial area of mice activated sensory neurons in the vomeronasal system. They tested the activity of extracts from glands in the head region, which ultimately led to the identification of 7 kDa peptide, which they named ESP1 (Kimoto et al. 2005). They went on to show that the gene encoding ESP1 was a member of a family of at least 38 related genes in mice, and 10 in rats (Kimoto et al. 2007). ESPs are produced by several glands, in addition to the extraorbital lacrimal glands, including salivary and Harderian glands. The finding that some ESPs are expressed in a sex- and strain-dependent manner, suggests that they could convey information about gender and individual identity, although their behavioral role is unknown (Kimoto et al. 2007).

#### 6.4 PHEROMONAL DETECTION

The species specificity of pheromonal signals is reflected in the high rate of evolutionary change of the signals and the chemosensory systems responsible for their detection. Probably the most significant of these changes was the transition from aquatic to terrestrial environments, due to their different physiochemical nature. With the evolution of a terrestrial lifestyle came the possibility to exploit the large range of airborne chemosignals by the ciliated cells of what came to be the main olfactory system. However, sensitivity to water-soluble, but relatively involatile chemosignals of the aquatic environment was not lost. Instead, the microvillar cells of the ancestral olfactory organ became largely segregated in an anatomically separate organ, in early terrestrial vertebrates, known as the vomeronasal organ (VNO), at the same time that the main olfactory system was adapting to sense airborne volatile stimuli. However, the detailed picture is considerably more complicated (Eisthen 2004) and the division between cell types is not absolute. Although the majority of olfactory sensory neurons (OSNs) in the mammalian main olfactory epithelium (MOE) are ciliated and express olfactory receptors (ORs), there are also microvillar cells that appear to form a distinct chemosensory system (Elsaesser et al. 2005).

For many years, the established view has been that these two chemosensory systems were not only anatomically distinct, but also functionally separate. The MOE was thought to detect volatile odors for general odor perception and learning. In contrast, the VNO was specialized for the detection of pheromonal signals affecting physiology and behavior, via a separate and relatively direct neural pathway. However, more recent studies have shown that both OSNs and vomeronasal sensory neurons (VSNs) can respond to the same chemical stimuli, and both sensory systems send projections to brain areas that are involved in mediating pheromonal responses (Brennan and Zufall 2006). Furthermore, the simple story of a distinction between the roles of the main olfactory and vomeronasal systems has become considerably more complicated by the discovery of specialized subsystems within both the main olfactory system and the vomeronasal system.

#### 6.4.1 VOMERONASAL SYSTEM

The vomeronasal system is often regarded as having a role exclusively in pheromonal detection. However, this is certainly not true in nonmammalian vertebrates, as the VNO is used to detect predator and prey odors in many reptiles (Halpern and Martínez-Marcos 2003), and may have a similar role in some mammals. The VNO is a blind-ended tubular structure situated in the nasal septum and connected to the nasal and/or oral cavities via a narrow duct (Døving and Trotier 1998). The sensory epithelium containing the VSNs is found on the medial side of the organ, which respond to stimuli that are pumped into the lumen of the organ following direct physical contact with a scent source. The mechanism of this pumping action is likely to vary among species. In rodents, such as hamsters and mice, the VNO is tightly enclosed in a cartilaginous capsule. Changes in the blood flow to a large laterally positioned blood vessel cause pressure changes in the VNO lumen, resulting in chemosignals being pumped into the organ, along with mucus (Meredith and O'Connell 1979). This vascular pumping mechanism is activated by the sympathetic nervous system in situations of behavioral arousal (Meredith 1994). However, in other species, uptake of stimuli into the VNO is thought to be associated with a behavior known as flehmen, involving curling of the upper lip and facial grimacing, which can often be observed in ungulates and felines following direct contact with a scent source.

Although the VNO is undoubtedly specialized for the detection of involatile stimuli, there is still some doubt about whether it responds to volatile airborne stimuli. Many pheromonal stimuli that are sensed by the VNO are small, volatile molecules, and they act as stimuli for VSNs *in vitro* (Leinders-Zufall et al. 2000). However, their binding to lipocalins, such as MUPs, could be required to transport them into the VNO. Functional magnetic resonance imaging of the accessory olfactory bulb (AOB), which receives the input from the VNO, in anaesthetized mice has revealed robust changes in activity in response to urine odors delivered via the nasal airstream (Xu et al. 2005). However, this activation of the AOB could have occurred via a centrifugal pathway activated by main olfactory input, rather than being a direct sensory response (Martel and Baum 2007).

#### 6.4.1.1 Vomeronasal Receptors

Two classes of vomeronasal receptors are expressed by spatially distinct populations of VSNs. Latest analyses of the mouse genome has revealed 187 functional genes for V1Rs (Grus et al. 2005), which are expressed by VSNs in the apical layer of the vomeronasal epithelium. A further 70 functional receptors of the V2R class have been identified, which are expressed by VSNs in the basal layer of the sensory epithelium (Shi and Zhang 2007). This surprising number of functional vomeronasal receptors indicates that there are likely to be a wide variety of chemosensory signals sensed by the vomeronasal system that remain to be identified. However, the vomeronasal receptor repertoire of mice and perhaps other rodents is not representative of all mammals. Many mammals have a much more restricted range of V1Rs and no functional V2Rs at all (Table 6.2).

Electrophysiological recordings and calcium imaging have revealed that the V1R and V2R classes of vomeronasal receptor respond to different classes of stimuli. V1R-expressing VSNs typically respond to small, volatile chemosignals, including the testosterone-dependent volatiles of male mouse urine (Figure 6.1) (Leinders-Zufall et al. 2000). They are also likely to respond to sulfated steroids that have recently been found to activate a large proportion of VSNs in the vomeronasal epithelium (Nodari et al. 2008). In contrast, the V2R-expressing population of VSNs is stimulated by a variety of protein and peptide stimuli, including MUPs, major histocompatibility complex (MHC) peptides, and ESPs (Leinders-Zufall et al. 2004; Chamero et al. 2007; Kimoto et al. 2007). Simultaneous recordings from large populations of VSNs in VNO slices have shown that natural stimuli, such as urine and tear secretions, contain a wealth of information about sex and individual identity, which could potentially be extracted by combinatorial analysis (Figure 6.2) (Holy et al.

## **TABLE 6.2**

A Comparison of the Number of Genes and pseudogenes for Major Urinary Proteins (MUPs), Vomeronasal Receptor Class 1 (V1Rs), and Class 2 (V2Rs) in a Range of Mammalian Species That Have Been Identified by Comparative Genomic Analysis

	MUPs	V2Rs	V1Rs
Human	0(1)	0(7)	5 (115)
Mouse	20 (18)	0(7)	187 (121)
Rat	9 (13)	70 (139)	106 (66)
Dog	1 (0)	59 (109)	8 (33)
Cow	1 (0)	0 (5)	40 (45)
Opossum	6 (1)	79 (72)	98 (30)

*Sources:* Adapted by permission from Cold Spring Harbor Laboratory Press and Macmillan Publishers Ltd: [Nature] from Shi, P. and Zhang, J., *Genome Res.*, 17, 166–74, copyright (2007) and Chamero, P. et al. *Nature*, 450, 899–902, copyright (2007).

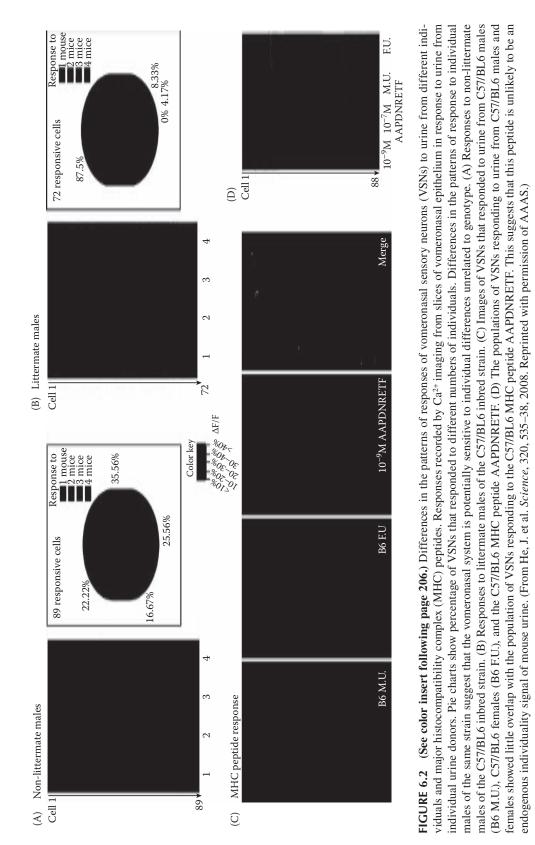
2000; Kimoto et al. 2007; He et al. 2008; Nodari et al. 2008). Although, the extent to which the vomeronasal system processes information in this way is not known.

As would be expected of a pheromonal detection system, the responses of VSNs are highly sensitive. V1R-expressing VSNs typically respond to concentrations of urinary volatiles, such as (R,R)-3,4-dehydro-exo-brevicomin (DB) and (S)-2-sec-butyl-4,5-dihydrothiazole (SBT), with thresholds of  $10^{-10}$  to  $10^{-11}$  M (Leinders-Zufall et al. 2000). V2R-expressing VSNs appear to be even more sensitive, responding to MHC peptides at the astonishingly low concentration of 10<sup>-13</sup> M (Leinders-Zufall et al. 2004). VSNs respond more selectively than classical OSNs (Figure 6.1), and maintain their selectivity as the stimulus concentration is increased (Leinders-Zufall et al. 2000). Vomeronasal transduction differs from the classical OSN transduction mechanism. VSN transduction appears to involve the phospholipase 2 signaling pathway and transient receptor potential channels of the TRPC2 variety, in the apical microvilli of VSNs (Holy et al. 2000; Leypold et al. 2002; Stowers et al. 2002). However, the responses of V2R-expressing VSNs to MHC peptides are unaffected in TRPC2 knockout mice, implying that they use a different and so far unknown transduction mechanism (Kelliher et al. 2006). Earlier reports of maintained firing rate during current injection into VSNs suggested that they failed to show significant adaptation (Holy et al. 2000). However, more recent studies have shown that VSNs do show adaptation to maintained or repeated stimulus presentation mediated by a Calcium-calmodulin-dependent feedback on TRPC2 cation channels (Spehr et al. 2009).

## 6.4.1.2 Vomeronasal Neural Pathways

VSNs project their axons to the AOB where they synapse with the primary dendrites of mitral cell projection neurons in glomerular structures. V1R and V2R classes of VSN, which are segregated in apical and basal regions of the vomeronasal epithelium, project separately to anterior and posterior subdivisions of the AOB, respectively (Halpera and Martinez-Marcos 2003). Recently, a third subsystem within the AOB has been identified (Ishii and Mombaerts 2008). A subpopulation of V2R-expressing VSNs coexpress nonclassical class I MHC genes. This population of VSNs is located in the deeper sublayer of the basal zone of the sensory epithelium and project to the posterior subdomain of the posterior subdivision of the AOB (Ishii and Mombaerts 2008). However, the significance of this tripartite organization of the AOB remains unclear.

Genetically manipulated mice in which VSNs that express different V1Rs have been labeled with different fluorescent markers has provided the first glimpse of the pattern of information flow within the anterior subdivision of the AOB (Wagner et al. 2006). This has revealed that AOB mitral cells



send a branched primary dendritic tree to sample information from glomeruli that receive input from different, but closely related V1R receptor types. These findings suggest that the integration of information from different receptor types is already occurring at the level of the AOB. This is consistent with recordings of AOB mitral cell activity from freely behaving mice, which found highly selective responses of individual neurons to specific combinations of sex and strain identity (Luo et al. 2003). A similar convergence of information at the level of the AOB is evident in the suppression of mitral cell responses to a mixture of male and female urine, compared to their responses to male or female urine presented individually (Hendrickson et al. 2008).

AOB mitral cells appear to send a distributed projection to the medial amygdala (MeA), posteromedial cortical amygdala (PMCoA), bed nucleus of the stria terminalis, and the bed nucleus of the accessory olfactory tract (von Campenhausen and Mori 2000). From these regions, vomeronasal information can gain direct access to the hypothalamic areas involved in the generation of a coordinated endocrine, autonomic, and behavioral output. Male and female chemosignals activate different subpopulations of neurons in the MeA, which can be identified on the basis of their homeodomain gene expression (Choi et al. 2005). Retrograde neural tracing in male mice showed that the MeA neurons that responded to female chemosignals provided input to areas of the hypothalamus involved in mating behavior. In contrast, MeA neurons that responded to male chemosignals projected to areas of the hypothalamus known to be involved in mediating defensive/aggressive behavior. Importantly, these male-responsive MeA neurons also sent antagonistic projections to the hypothalamic areas controlling reproductive behavior (Choi et al. 2005). This suggests that female pheromonal input normally drives mating behavior in males, but in the presence of male pheromones from a potential competitor, reproductive behavior is inhibited and defensive aggressive behavior promoted. Thus, there appear to be antagonistic interactions between male and female chemosensory information at the level of hypothalamic output as well as in the level of the AOB.

#### 6.4.1.3 Behavioral Effects of Vomeronasal Dysfunction

The importance of the vomeronasal system in influencing behavior has been demonstrated by experiments in which the VNO has been physically ablated in genetically normal mice, or vomeronasal transduction disrupted in genetically manipulated mice lacking TRPC2 ion channel function. A common finding across these studies is that the removal of vomeronasal function abolishes the aggressive responses that both male and lactating female mice normally show in response to a male intruder (Maruniak et al. 1986; Leypold et al. 2002; Stowers et al. 2002). This is consistent with the role of the VNO in detecting volatile and involatile male urinary constituents that elicit aggressive behavior (Novotny et al. 1985; Chamero et al. 2007).

There appear to be significant species differences in the importance of vomeronasal sensation for male sexual behavior. Forty percent of male hamsters show severe deficits in sexual behavior, following section of their vomeronasal nerves (Licht and Meredith 1987). The effects were particularly severe in sexually naïve males, with significant impairment of their ability to mate. However, sexually experienced males were much less affected, as their mating behavior could be maintained by main olfactory input that had become associated with mating during their previous sexual experience. In male mice, vomeronasal ablation prevents the normal rise in luteinizing hormone levels in response to female chemosignals. Male sexual behavior is not prevented in mice lacking vomeronasal function, suggesting that the pheromonal cues mediated by the main olfactory system may play an important role (Keller et al. 2009). Notably, TRPC2 knockout mice that have severely impaired vomeronasal function still show sexual behavior directed toward females, but also mount other males, rather than behaving aggressively toward them (Maruniak et al. 1986; Leypold et al. 2002; Stowers et al. 2002).

Physical lesions of the VNO impair lordosis behavior in female mice (Keller et al. 2006), suggesting that pheromones sensed by the vomeronasal system also play an important role in female sexual behavior (Keller et al. 2009). However, once again, the behavioral deficits of TRPC2 knockout mice appear to differ from the effects of physical lesions of the VNO. Dulac reported that TRPC2 knockout female mice showed significantly higher levels of malelike sexual behavior, including ultrasonic vocalization and mounting of other females (Wysocki and Lepri, 1991; Kimchi et al. 2007). This would suggest that sex-specific behavioral patterns of male and female mice are at least partly dependent on ongoing sensory input rather than being developmentally determined. But, other groups have not reported such effects, and both male and female mice with physical VNO lesions are capable of discriminating sexual identity of urine odors (Keller et al. 2009). The differences that have been reported between the behavioral effects of physical VNO lesions and knockout of the TRPC2 gene might arise due to developmental effects of the knockout, or due to the presence of VSNs that do not use the TRPC2 transduction pathway (Kelliher et al. 2006).

## 6.4.2 MAIN OLFACTORY SYSTEM

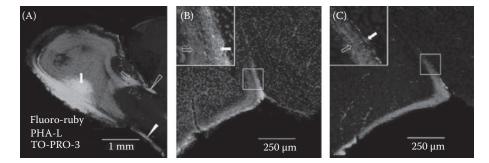
Although previously often overlooked, it has been known for many years that not all pheromonal responses are mediated by the vomeronasal system. For example, the mammary pheromone that guides nipple search behavior of rabbit pups is still effective following VNO lesion (Hudson and Distel 1986b). Similarly, the boar sexual attractant pheromone is still effective in eliciting standing behavior following VNO lesions in sows (Dorries et al. 1997). Instead, these pheromonal effects and many others are likely to be mediated by the main olfactory system. The main olfactory system has traditionally been thought to function as a pattern recognition system, associating patterns of activity across broadly tuned receptors into a representation of the complex odorant mixtures that make up natural odors. The emphasis has been very much on the role of learning in the piriform cortex in forming these odorant representations and associating them with their context and an appropriate behavioral response (Wilson and Stevenson 2003). This provides considerable flexibility to the main olfactory system in its ability to respond to novel odors, but does not really fit with a role in mediating innate responses to specific pheromonal stimuli. However, it is becoming increasingly apparent that the main olfactory system is not a unitary sensory system, but is composed of a number of functionally specialized subsystems that might be involved in pheromonal detection.

Among these main olfactory subsystems, OSNs expressing members of the trace amine receptor (TAAR) family have been found in the mouse MOE and can respond to volatile amines that are found in mouse urine (Liberles and Buck 2006). Another subpopulation of OSNs are distinguished by their guanyl cyclase-dependent transduction pathway. At least some of this population have been shown to respond to the peptides guanylin and uroguanylin, which are also found in mouse urine (Leinders-Zufall et al. 2007), although what pheromonal role they might perform is still unknown. Somewhat more surprising is the finding of a subpopulation of OSNs that respond to involatile MHC peptides. In a challenge to the dogma that the MOE only responded to volatile odors carried in the nasal airstream, Spehr et al. (2006) showed that the nonvolatile fluorescent dye, rhodamine, gained access to a large extent of the MOE following direct physical investigation of a rhodamine-painted conspecific. This suggests that other nonvolatile peptides, and possibly even proteins, could gain access to the MOE of mice following direct investigation of a stimulus.

The functions of the vomeronasal and main olfactory systems are more integrated than previously thought (Zufall and Leinders-Zufall 2007). The same chemosignals can act as stimuli for both OSNs and VSNs with low response thresholds typical for pheromonal detection. The mouse MOE responds to urinary volatiles, such as heptanone, at concentrations of 10<sup>-10</sup> M (Spehr et al. 2006), similar to the sensitivity of V1Rb2 expressing VSNs (Leinders-Zufall et al. 2000). The responses of the two systems to MHC peptides are also highly sensitive, with responses at 10<sup>-10</sup> M for OSNs (Spehr et al. 2006), and down to 10<sup>-13</sup> M for VSNs (Leinders-Zufall et al. 2004). Furthermore, trans-synaptic tracing of the afferent connections of neurons expressing luteinizing hormone-releasing hormone have revealed that both the main olfactory system and the vomeronasal system provide input to these hypothalamic neurons that regulate reproductive physiology and behavior (Boehm et al. 2005; Yoon et al. 2005). By its very nature, input from releaser and primer pheromones is likely to mediate innate responses via specialized neural pathways, separate from the general odor-sensing pathway of the main olfactory system. Indeed, part of the MOB has been found to mediate innate responses to odors (Kobayakawa et al. 2007). Ablation of OSN input to the dorsal zone of the MOB, using targeted expression of the diphtheria toxin gene, disrupted the innate aversive response of mice to rancid food odors and to predator odors. This failure to show an innate aversive response to the odors was not due to an anosmia, as the mice were still able to detect the odors and could be trained to show conditioned aversion to them. Although these are not pheromonal effects, they demonstrate that information about innate odor responses is handled by a separate pathway to that of learned odor responses in the MOB (Kobayakawa et al. 2007).

Until recently, the AOB and MOB were thought to project to separate brain areas. Even their projections to the amygdala were thought to target different nuclei. The AOB projects to the MeA and PMCoA, which together are often referred to as the vomeronasal amygdala (von Campenhausen and Mori 2000). These areas, in turn, project to medial regions of the hypothalamus involved in the control of reproductive and social behavior. The MOB projects to the neighboring anterior cortical and posterolateral cortical regions of the amygdala. Electrophysiological recording in hamsters has found that information from the main olfactory system and vomeronasal system converges on individual neurons in the MeA (Licht and Meredith 1987).

This influence of the main olfactory input on the MeA was thought to be mediated by indirect intra-amygdala connections. However, a recent study using anterograde tracing has identified a previously neglected, direct projection from the MOB to the MeA in mice and rats (Figure 6.3) (Kang et al. 2009). This potentially provides a more direct pathway by which main olfactory input could control reproductive and social behavior. Retrograde tracing from the MeA revealed that these projections originated from a subpopulation of mitral and tufted (M/T) neurons located mainly in the ventral region of the MOB. Interestingly, these retrogradely labeled M/T neurons in the MOB of female mice responded to chemosignals from male mice, but not to chemosignals from other female mice, or to a predator odor. These M/T neurons were in a similar location to the ventrally located MOB glomeruli that receive input from TRPM5-expressing OSNs (Lin et al. 2007). Moreover, M/T neurons in this region of the MOB respond to social chemosignals present in male urine, such as the urinary attractant MTMT (Lin et al. 2007), and suggest that this is a likely pathway for many pheromonal effects on reproductive behavior that are mediated by the main olfactory system.



**FIGURE 6.3** (See color insert following page 206.) Convergence of input from the ventral main olfactory bulb (MOB) and the accessory olfactory bulb (AOB) onto the medial amygdala (MeA) of the female mouse. (A) Location of injections of anterograde tracer into the ventral MOB in green, shown by filled arrow, and AOB in red, shown by open arrow. Convergence of projections from MOB (green, filled arrow) and AOB (red, open arrow) onto neighboring laminae in the anterior region of the MeA (B) and posterodorsal subdivision of the MeA (C). (Reprinted from Kang, N., Baum, M.J., and Cherry, J.A., *Eur. J. Neurosci.*, 29, 624–34, 2009. With permission from Wiley-Blackwell.)

## 6.5 PHEROMONAL EFFECTS ON BEHAVIOR

Chemical signals that elicit a specific and immediate behavioral effect are known as releaser pheromones. Pheromones that elicit longer-term effects on endocrine state or development are termed primer pheromones. However, pheromonal signals can have different effects in different contexts. For example, testosterone-dependent constituents of male mouse urine, including DB, SBT, E,E- $\alpha$ -farnesene, E- $\beta$ -farnesene, and 6-hydroxy-6-methyl-3-heptanone, are all effective individually in accelerating puberty in prepubertal female mice (Novotny et al. 1999). A mixture of two of these compounds, DB and SBT, is also effective in inducing and synchronizing estrus cycles in adult females (Ma et al. 1999), and also has a releaser pheromonal effect in eliciting aggression from males or maternal females, when presented in the context of an intruder male (Novotny et al. 1985). It is, therefore, more useful to classify the effect of a pheromone as being releaser or primer, rather than applying the terms as labels to particular substances.

As our understanding of vertebrate chemical signaling has advanced, new classes of chemosignals have been identified that do not fit the original definition of a pheromone (Wyatt 2003). This has led some researchers to propose new categories of pheromonal effects (Wysocki and Preti 2004). The term signaler pheromone has been used for chemosignals conveying information about the producer that might bias behavioral choices, without mediating a definite response; for instance, chemical signals that convey information about individual identity that are used in territorial marking. A further category of modulator pheromone has been used to describe the effects of chemical signals that alter mood, such as appeasement pheromones that are reportedly produced by nursing females and have a calming effect on their offspring, or the anxiety-promoting effects of alarm pheromones. However, these new classifications are not as widely accepted as the original distinction between primer and releaser effects. An alternative, and potentially more useful classification has been proposed by Wyatt (2009), which distinguishes between pheromones that mediate innate responses and "signature odors," such as individuality signals, that convey information and for which learning determines the nature of the response.

## 6.5.1 SEXUAL ATTRACTANT PHEROMONES

Attractant pheromones are often used to arouse, attract investigation, and release specific behavioral responses from conspecifics. One well-known example is the boar sexual attractant pheromone, which has even been exploited commercially as a test for sow receptivity. Boar saliva contains high levels of the androgen derivatives  $5\alpha$ -androst-16-en-3-one and  $5\alpha$ -androst-16-en-3-ol. These steroids are bound and concentrated in the saliva by proteins SAL1 and SAL2, which are members of the lipocalin family of ligand-binding proteins (Loebel et al. 2000). When sexually aroused, boars salivate profusely and foam at the mouth, which disperses these volatile pheromones in the air. The  $5\alpha$ -androst-16-en-3-one and  $5\alpha$ -androst-16-en-3-ol act as releaser pheromones to attract receptive sows and elicit a specific mating posture, known as standing, which allows mounting by the boar (Dorries et al. 1997).

Another example of a sexual attractant is aphrodisin, a 17 kDa protein found in the vaginal fluid of female hamsters, which elicits mounting behavior in sexually naïve, male hamsters. Aphrodisin is also a member of the lipocalin family of ligand-binding proteins, although it is still unclear whether synthetic aphrodisin that lacks its endogenous ligand is effective in stimulating mounting behavior (Briand et al. 2004). Mouse urine also contains attractive chemosignals that promote investigation by opposite sex conspecifics. The urinary constituents responsible for the innate attractiveness of urine appear to be involatile and likely to be MUPs, which are also lipocalins (Ramm et al. 2008). Urinary volatiles, such as the MTMT produced by male mice, have also been reported to have attractant properties. Although synthetic MTMT was relatively ineffective in isolation, it increased the investigation time of females when added to urine (Lin et al. 2007).

# 6.5.2 RABBIT MAMMARY PHEROMONE

Other pheromones that elicit a strong behavioral attraction are the nipple guidance pheromones. The best understood example is the rabbit mammary pheromone, but similar pheromonal stimuli may be of importance in guiding offspring to nipples and facilitating nursing in most mammals, including humans. Rabbits have an extreme form of maternal care, in which they only make brief 4–5 min nursing visits to their pups once a day. During this short period, the rabbit pups are guided to the mother's nipples by a pheromone produced by the nipples and which is present in the milk (Hudson and Distel 1986a). This pheromone elicits a specific pattern of behavior known as nipple searching, in which the pup's forelimbs are splayed laterally and the head makes rapid side-to-side searching movements, scanning the mother's ventrum. The gradient of mammary pheromone guides the pup's nose to the nipples to which it can attach on the basis of somatosensory cues (Distel and Hudson 1985).

Analysis of the volatile constituents of rabbit milk showed that a single constituent, 2-methylbut-2-enal, was capable of eliciting full nipple search behavior (Schaal et al. 2003). Unusually for mammalian pheromones, the synthetic compound was also effective when presented on a glass rod, outside the normal suckling context. The effect of the mammary pheromone to releases nipple search response appears to be automatic in young rabbit pups, irrespective of whether or not they have recently fed. However, in five-day-old pups, its effectiveness was found to decline immediately after suckling, showing that the pheromone's influence over behavior less-ened during development to become modulated by prandial state (Montigny et al. 2006).

# 6.5.3 MOUSE AGGRESSION PHEROMONES

A mixture of the testosterone-dependent urinary volatiles DB and SBT are able to elicit aggressive behavior from male mice when added to castrated male urine (Novotny et al. 1985), consistent with their response being mediated by the V1R-expressing class of VSN (Leinders-Zufall et al. 2000). Recently, it has been reported that the nonvolatile fraction of male mouse urine is also effective in eliciting male aggression (Chamero et al. 2007). Analysis of this fraction revealed this involatile aggression-promoting pheromone to be a MUP. Furthermore, a synthetic MUP was able to elicit aggression and stimulate V2R-expressing VSNs, even in the absence of the aggression-promoting volatiles DB and SBT (Chamero et al. 2007; Kimoto et al. 2007). Therefore, MUPs and the testosterone-dependent volatile that they bind act via separate vomeronasal receptor pathways to elicit aggressive/defensive behavior in mice.

# 6.5.4 ALARM PHEROMONES

Under stressful conditions, such as elevated levels of carbon dioxide, mice release alarm pheromones that elicit freezing behavior in other mice. These alarm pheromones are volatile and water soluble, but their chemical identity is unknown. They are sensed by chemosensory neurons in the Grueneberg ganglion, as the freezing response is abolished in mice with section of sensory nerve from the ganglion (Brechbühl et al. 2008).

# 6.5.5 PHEROMONES AND LEARNING

Recent evidence suggests that some pheromones can be innately rewarding and promote associative learning. Naïve female mice do not normally show a preference for investigating volatile urinary odors from males. However, they are innately attracted to the involatile (presumably protein) constituents of male mouse urine, and will spend significantly more time investigating them than those from female urine or urine from castrated males. These urinary proteins are not only innately attractive to females, but also promote learning of the volatile urinary odors with which they are associated (Moncho-Bogani et al. 2005; Ramm et al. 2008). Surprisingly, this prior experience with the nonvolatile constituents does not generally increase the attractiveness of the urinary volatiles of all males, but only the attractiveness of the individual male's volatiles to which the females were exposed (Ramm et al. 2008). Similarly, exposure of rabbit pups to an artificial odor that has been paired with the mammary pheromone without suckling, will condition the full nipple search response to the artificial odor when subsequently presented alone (Coureaud et al. 2006). Such findings are consistent with certain pheromones being intrinsically rewarding, which not only promotes further investigation of the pheromonal stimulus, but also potentially reinforces the pheromonal effect due to the learned response to associated contextual cues.

# 6.6 CHEMICAL SIGNALS OF INDIVIDUAL IDENTITY

Mammals release an enormous variety of molecules into the environment that contribute to their chemical profile, and which could potentially be used to recognize the individuality of the producer. But which, if any, of these can usefully thought of as pheromones? This remains a controversial area, with many researchers in this field deliberately avoiding the use of the term. Chemicals that convey information about individual identity do not generally elicit a direct response, but provide information that may bias the current response, or a future response of an individual. Such biasing effects are often associated with learning and as they are dependent on both past and present context, they do not meet the conventional definition of a pheromone. Nevertheless, the finding of specific classes of chemosignal, and sensory responses that appear to be adapted to convey individual information, suggests that these "signature odors" are likely to have important influences on behavior (Brennan and Kendrick 2006).

## 6.6.1 MAJOR URINARY PROTEINS AND TERRITORIAL MARKING

Territorial behavior is seen in a wide variety of species in which individuals compete to monopolize desirable territories and resources. Many mammals deposit scent cues around their environment, advertising their presence to competitors and to signal their reproductive fitness to potential mates. This is perhaps best understood in mice, in which dominant males deposit urine marks throughout their territory, and especially along boundaries and access routes (Hurst and Beynon 2004). Like many other species that use urine marking, mice excrete large quantities of protein in their urine. Typically, 99% of the protein content of the urine is made-up of MUPs, members of the lipocalin family of ligand-binding proteins (Beynon and Hurst 2003). The concentration of MUPs is four to five times higher in male mouse urine than that of females, and some MUP variants are found only in males (Robertson et al. 1997).

MUPs bind certain volatile urinary constituents, including the testosterone-dependent male mouse pheromones DB and SBT, which have been shown to have pheromonal effects on the female reproductive state and the initiation of male aggression. MUPs are highly stable in the environment and act as a reservoir for the volatile ligands, prolonging their release over a period of days (Hurst et al. 1998). These characteristics make MUPs ideally suited as a territorial marker. Not only does the release of volatiles attract investigation to the urine mark, advertising the presence of the nonvolatile protein component, but the amount of volatiles being released from the mark is also a reliable indicator of the age of the urine mark. When a resident male comes across a urine mark of a rival male, the resident deposits his fresh urine mark next to the aging mark of his competitor. This countermarking behavior depends on the male being able to make physical contact with the involatile protein components in the urine mark, presumably MUPs that are being sensed by the VNO (Sherborne et al. 2007). The assessment of the relative ages of urine marks therefore, provides females with an honest signal of the competitive ability of males to dominate their territory without the males engaging in potentially damaging direct confrontation (Humphries et al. 1999).

In order to use urine marking as an indicator of the competitive fitness, urine marks have to be associated with the individual that produced them. In addition to their physiochemical properties that make MUPs ideal as territorial markers, MUPs are highly polymorphic and a wild mouse will produce an individual profile of different MUP types capable of conveying individual identity. Individual mice captured from the wild produce between 5 and 15 variants from the polymorphic MUP family, the profile of which is specific for an individual (Robertson et al. 1997). Moreover, the recognition of urine marks can be influenced by the addition of an artificially produced recombinant MUP to change their MUP profile (Hurst et al. 1998; Robertson et al. 2007). MUPs without bound ligands have been shown to act as stimuli at V2R-expressing VSNs (Chamero et al. 2007; Kimoto et al. 2007). This role of these VSNs in detecting individual MUP variants is consistent with genomic analysis that has found an association between the number of genes for MUP isoforms and for V2Rs in certain species (Table 6.2). However, although genomic analysis has revealed expansions of the MUP gene family in mice, rats, horses, and gray lemurs, many species have only a single MUP isoform and appear to be unable to use MUPs to encode individual identity (Logan et al. 2008).

# 6.6.2 MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)-ASSOCIATED CHEMOSIGNALS

In identifying chemosensory signals of individual identity, most attention has focused on genes of the MHC, which determine the recognition of self from non-self by the immune system. This is a highly polymorphic family of genes, therefore individuals in the wild generally have different MHC types in addition, but unrelated, to other genetic differences such as MUP genotype.

# 6.6.2.1 Major Histocompatibility Complex (MHC)-Associated Volatiles

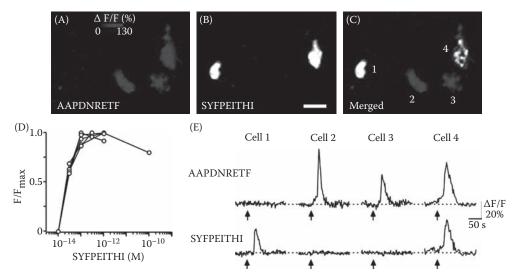
Many years of research have shown that both trained and untrained mice can discriminate the volatile urine odors of MHC-congenic mice that differ genetically only at the H2 region of their MHC (Yamaguchi et al. 1981; Penn and Potts 1998b). Urine samples from MHC-congenic mice have consistently different proportions of volatile carboxylic acids (Singer et al. 1997) and elicit significantly different patterns of activity in the MOB (Schaefer et al. 2002). The ability of mice to discriminate MHC-congenic urine odors has been reported as being related to polymorphism in their peptidebinding groove (Carroll et al. 2002). However, genetically identical inbred mice have a significant variability in the proportion of volatile urinary components, suggesting that nongenetic factors, such as nutrition and environmental condition, also have significant effects on individual urine odor (Röck et al. 2007). Despite several theories having been proposed, no mechanism has been established by which MHC genotype could affect metabolic pathways to account for the reported quantitative differences in urinary volatiles.

# 6.6.2.2 Major Histocompatibility Complex (MHC) Peptides

The H2 region of the mouse MHC codes for MHC proteins of classical class I type, which are expressed on the cell membrane of nearly all nucleated cells in vertebrates. Their immunological role is to bind peptides resulting from proteosomal degradation of endogenous and foreign proteins, and present them at the cell surface for immune surveillance (Boehm and Zufall 2006). The specificity of peptide binding is determined by the position of bulky amino acid side chains, known as anchor residues, which fit into binding pockets in the MHC class I peptide-binding groove. Therefore, individuals with different MHC type will bind different subsets of peptides having anchor residue positions that mirror the polymorphic differences in the peptide-binding groove of their MHC class I proteins. For example, MHC class I proteins of C57BL/6 inbred mice (H-2b haplotype) preferentially bind peptides having asparagine (N) at position 5, such as AAPDNRETF, whereas MHC class I proteins of the BALB/c inbred strain (H-2d haplotype) preferentially bind peptides with tyrosine (Y) at position 2, such as SYFPEITHI. As the anchor residue structure of MHC-peptide ligands reflect the peptide-binding cleft of the MHC class I peptide that bound them, they could potentially function as robust signals of MHC identity.

This hypothesis has been investigated using electrophysiological recording and calcium imaging of slices of mouse vomeronasal epithelium. Responses to synthetic peptides possessing the characteristic features of MHC-peptide ligands have been reported at concentrations from  $10^{-9}$  to  $10^{-13}$  M (Chamero et al. 2007; He et al. 2008), although the percentage of MHC-peptideresponsive cells varied widely among the studies. Individual VSNs responded selectively to synthetic BALB/c-type (SYFPEITHI) or C57BL/6-type (AAPDNRETF) peptides (Figure 6.4) (Leinders-Zufall et al. 2004). VSN responses were abolished when the bulky anchor residues were substituted with alanine residues, which lack a side chain. Furthermore, the position of the anchor residues was shown to be critical. Changing the position of the anchor residues abolished the responses of VSNs, whereas the selectivity of responses from individual VSNs were not affected when the anchor residues were left unchanged, but the intervening sequence of amino acids was varied. Most VSNs responded selectively to synthetic peptides of either BALB/ctype or C57/BL6-type, however, a small proportion responded to both peptides (Leinders-Zufall et al. 2004), suggesting the expression of more than a one V2R receptor type per VSN. But only a limited amount of evidence has been found for such coexpression (Martini et al. 2001). Future experiments testing a wider range of MHC-peptide types will be required to determine whether individual VSNs respond to specific combinations of MHC peptides that could encode individual identity.

Calcium imaging of MHC-peptide sensitive VSNs revealed them to be located in the basal layer of the vomeronasal epithelium, colocalizing with VSNs expressing the V2R class of vomeronasal receptor (Leinders-Zufall et al. 2004). These receptors possess a large extracellular N-terminal domain, possibly involved in binding proteins or peptides and are coexpressed with atypical MHC proteins of the Ib class (Ishii et al. 2003; Loconto et al. 2003). These nonclassical MHC Ib proteins have only been found expressed in the VNO and form a receptor complex with V2Rs and  $\beta$ -microglobulin, suggesting that they might have a specific chemosensory function (Loconto et al. 2003). Certain combinations of MHC Ib proteins are coexpressed with particular V2Rs, which



**FIGURE 6.4** Vomeronasal sensory neurons (VSNs) respond to major histocompatibility complex (MHC) peptides. Ca<sup>2+</sup> imaging in slices of vomeronasal epithelium of responses from four VSNs in response to synthetic MHC peptide of (A) C57/BL6-type AAPDNRETF (pseudocolored dark gray), (B) BALB/c-type SYFPEITHI (pseudocolored light gray), (C) merged image. (D) Highly sensitive responses of VSNs responding to BALB/c peptide SYFPEITHI. (E) Selectivity of responses of VSNs shown in A, B, and C to synthetic peptides of C57/BL6-type AAPDNRETF and BALB/c-type SYFPEITHI. (From Leinders-Zufall, T. et al. *Science*, 306, 1033–37, 2004. Reprinted with permission of AAAS.)

could affect receptor specificity (Ishii et al. 2003). Sequence variability among the nine members of the nonclassical MHC Ib family is localized to the peptide-binding groove. But structural considerations suggest that they are unlikely to bind peptides (Olson et al. 2005) and their role in VSN function remains unknown.

It is becoming increasingly apparent that there is considerable overlap between stimuli that are sensed by the main olfactory and vomeronasal systems (Brennan and Zufall 2006). But, it is nevertheless surprising that responses to MHC-peptide ligands have also been recorded from the MOE (Spehr et al. 2006). Calcium imaging of individual OSNs in the MOE revealed that they respond selectively to MHC peptides down to 10<sup>-11</sup> M. This is one to two orders of magnitude higher than the threshold for MHC-peptide-responsive VSNs, which along with their lack of absolute dependence on anchor residues suggests that a different type of receptor may be involved. Whereas replacement of anchor residues with alanines abolished the responses of VSNs, it shifted the stimulus response curve of individual OSNs, although OSNs still failed to respond to the scrambled version of the peptide in which the position of the anchor residues had been changed (Spehr et al. 2006). Therefore, OSN responses to MHC peptides may be more dependent on the overall sequence of amino acids, rather than the position of the anchor residues. Such ability to recognize specific MHC peptides could theoretically confer the ability to detect peptides of pathogenic origin, and convey information about the health status of a conspecific, rather than information about genetic identity, although there is no evidence for this conjecture at present.

# 6.6.3 ROLE OF MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)-ASSOCIATED CHEMOSIGNALS IN NATURAL CONTEXTS

## 6.6.3.1 Mate Choice

Despite over 30 years of research, the importance of any influence MHC genotype might have on mammalian behavior remains unclear. An influence of MHC genotype on mate choice in mice was first reported by Boyse, and investigated in a series of further studies by Yamazaki and Beauchamp (Boyse et al. 1987). They reported a disassortative pattern of mating in which male mice preferred to mate with females of dissimilar MHC type, thus avoiding inbreeding. This influence of MHC type on mate choice depended on learning of kin odors in the nest environment, as it was substantially reversed by cross-fostering mouse pups onto MHC-dissimilar mothers (Yamazaki et al. 1988; Penn and Potts 1998a). However, many similar studies of mate choice have produced inconsistent and sometimes conflicting results (Jordan and Bruford 1998). This failure to consistently find a clear effect of MHC type is likely to be due to the difficulties inherent in studying such complex behavior as mate choice in a limited laboratory environment. For mate choice tests, mice are frequently restrained and deprived of the normal behavioral context in which they can assess the reproductive fitness of potential mates. Moreover, the use of congenic mice that only differ in MHC type removes much of the genetic variability that may normally contribute to mate choice decisions.

Disassortative mate preference has been observed in seminatural enclosures, in which colonies of mice produced fewer MHC homozygous offspring than expected from random matings (Potts et al. 1991). However, a recent large study that followed wild-derived mice, which were allowed to breed freely in a large outdoor enclosure, failed to find any evidence for an effect of MHC type (Sherborne et al. 2007). Rather, mate choice was related to MUP similarity, with a deficit in matings between individuals that shared both MUP haplotypes. An important point of this experiment was its use of mice bred from wild-captured individuals, which have considerably more genetic variability, especially with regard to MUP profiles, than inbred strains (Cheetham et al. 2009). More experiments will be required, using wild-derived mice in natural contexts, before the relative importance of MHC genotype, MUP profile, and general heterozygosity in mate choice decisions can be fully understood.

## 6.6.3.2 Mother–Offspring Interactions

Female mice are more likely to form communal nests with kin of MHC-similar genotype (Manning et al. 1992). Female mice also preferentially retrieved pups of similar MHC type to themselves, which had been removed from the nest and mixed with MHC-dissimilar pups (Yamazaki et al. 2000). Furthermore, mouse pups themselves appear to use MHC-related cues to learn the odor of their mother and siblings, as revealed by their preference for odors of maternal MHC type in an odor choice test (Yamazaki et al. 2000). These MHC influences on behavior could be largely reversed by cross-fostering, showing their dependence on learning of signature odors in the nest environment. This is consistent with the role of the main olfactory system in learning to recognize complex mixtures of odorants that make up individual odors, whether or not those are genetically determined.

## 6.6.3.3 A Behavioral Role for Major Histocompatibility Complex (MHC) Peptides?

MHC genotype has also been linked to mate recognition in the Bruce effect. This is a primer pheromonal effect in which exposure of a recently mated female mouse to urine from an unfamiliar male causes implantation failure and a return to estrus (Bruce 1959). However, the pregnancy-blocking effectiveness is also affected by individuality chemosignals present in the urine, as urine from the mating male is ineffective in blocking his mate's pregnancy. Both the Bruce effect and the recognition of the mating male are mediated by the vomeronasal system (Lloyd-Thomas and Keverne 1982; Ma et al. 2002). This ability of the female to recognize the urinary chemosignals of her mate is due to her learning their identity at mating, which subsequently inhibits the transmission of the pregnancy-blocking signal at the level of the AOB (Brennan and Zufall 2006). Congenic male mice, differing from the mating male only in their MHC genotype, were not recognized and blocked the pregnancy of recently mated females in a similar manner to an unfamiliar male of a different inbred strain (Yamazaki et al. 1983), suggesting an involvement of MHC-associated chemosignals.

The role of MHC peptides in this mate recognition has been investigated by testing the pregnancy-blocking effectiveness of urine from the mating male that had been spiked with synthetic MHC peptides of a different strain type (Leinders-Zufall et al. 2004). The addition of C57BL/6-type peptides to BALB/c male urine significantly increased its pregnancy-blocking effectiveness following mating with a BALB/c male. Conversely, the addition of BALB/c-type peptides to C57BL/6 male urine increased its effectiveness in blocking the pregnancy of females that had mated with a C57BL/6 male. This suggests that MHC-peptide ligands influence the individual signature of the mating male urine, providing support for the theory that they can convey information about individual identity via the vomeronasal system (Leinders-Zufall et al. 2004; Thompson et al. 2007).

However, a major problem with the hypothesis that MHC peptides convey individuality in the pregnancy block effect, or indeed any other biologically important context, is the failure to find them, to date, in any biological secretion, including male mouse urine. Furthermore, Ca<sup>2+</sup> imaging of vomeronasal epithelial slices has found that although some VSNs did respond to both the C57/BL6-type MHC peptide AAPDNRETF and to urine from C57/BL6 males (He et al. 2008), a significant number of VSNs only responded to one or the other, implying that this MHC peptide is not normally present in C57/BL6 male urine (Figure 6.2). Therefore, although MHC peptides may influence the pregnancy block effect, it is unlikely that they are the endogenous individuality signal present in urine.

## 6.7 HUMAN PHEROMONES

The idea that human physiology and behavior might also be influenced by pheromonal cues is a natural extension of the finding of pheromonal responses in other animals. But, despite a widespread research effort, it has been difficult to identify robust and reproducible effects. This doesn't necessarily mean that human pheromones don't exist, but complexities of modern human society may diminish their biological significance and make it difficult to identify consistent effects. Human axillary secretions from the armpit and genital regions provide a rich source of putative pheromonal signals. Microbial action on axillary apocrine secretions produces the complex mixture of odorants responsible for body odor, including androgen derivatives and volatile acids (Leyden et al. 1981). (E)-3-methyl-2-hexanoic acid (E-3M2H) is one of the major axillary secretions (Zeng et al. 1991). This is particularly interesting as it is bound by apolipoprotein D, a member of the lipocalin family of ligand-binding proteins that are often associated with pheromonal volatiles in other species (Zeng et al. 1996).

A VNO is present early in human fetal development, but appears to degenerate before birth, and the experimental evidence suggests that any residual structure that has been identified as the human VNO is nonfunctional (Meredith 2001). Not only does it lack the well-developed sensory epithelium found in the VNOs of other species, but also the sensory nerves to connect it to the brain (Witt and Hummel 2006). Furthermore the gene encoding the TRPC2 cation channel is a pseudogene in humans, the selection pressure on it having been relaxed around 23 million years ago, shortly before the separation of hominoids and Old World monkeys (Liman and Innan 2003; Zhang and Webb 2003). Analysis of the human genome reveals that almost all of the genes for vomeronasal receptors and transduction mechanisms are pseudogenes in humans. Therefore, any receptors for human pheromones are likely to be found in the MOE or possibly the Grueneberg ganglion, about which little is known, apart from a single report of its presence in humans. Possible candidates for human pheromonal receptors include members of the TAAR family of receptors (Liberles and Buck 2006). Four potentially functional V1R-like genes have also been identified in the human genome, of which hV1RL1 is expressed in the MOE, but whether it has any role in pheromonal communication is unknown (Rodriguez et al. 2000).

Perhaps the clearest pheromonal effects to detect in humans are primer effects on hormone levels and changes in physiological state, which are more easily measured and quantified than behavioral responses. 4,16-Androstadien-3-one, a compound present in male axillary secretions, has been found to increase levels of the hormone cortisol (Wyart et al. 2007), and to influence the frequency of luteinizing hormone pulses in females (Preti et al. 2003). Exposure to axillary secretions from other females has also been found to influence female menstrual cyclicity. Axillary odor stimuli from females in the late follicular and ovulatory phases of their menstrual cycle have been found to shorten and lengthen, respectively, the cycles of exposed females (Stern and McClintock 1998).

Whether pheromones can enhance sexual attraction in adult humans is a complex issue (Wysocki and Preti 2004). Effects of axillary secretions and synthetic putative pheromones on attractiveness ratings have been reported under laboratory conditions (Wysocki and Preti 2004). However, there has been a shortage of rigorous, placebo-controlled, double-blind studies on pheromonal effects on attractiveness and sexual activity in natural social situations. There are several problems with the interpretation of such studies, not least of which are the individual differences in the opportunities for and the nature of any social or sexual interactions. Imaging human brain activity has the potential to detect responses to putative pheromones, but these can be difficult to link to their behavioral effects due to the unnatural contexts and concentrations in which the putative pheromones are presented (Savic et al. 2001).

Although it is difficult to demonstrate convincing pheromonal effects on adult human behavior, the relative simplicity of human neonatal behavior potentially makes identifying the human equivalent of a mammary pheromone more feasible. Montgomery's glands, found in the areolar region around the nipple, produce a milky secretion, which has been suggested to contain a mammary pheromone that facilitates suckling. The breast odor of human mothers has been reported to attract newborn babies, and human babies spend significantly longer orienting toward human breast milk compared with formula milk (Marlier and Schaal 2005), similar to the attractant effects of the rabbit mammary pheromone. However, newborn babies show similar orientation responses to components of the mother's diet during gestation (Schaal et al. 2000), implying that it may be a learned response to maternal odors that the fetus was exposed to in utero. This potentially makes distinguishing an innate pheromonal response from a learned response to incidental maternal odors all the more difficult.

It is common knowledge that humans have individual odor signatures that can be discriminated by trained sniffer dogs and may also be influenced by MHC genotype. Overall, it seems that humans rate the odors of other individuals as being more pleasant if they share a few MHC alleles with the rater, ratha than either no matches or a high degree of similarity (Wedekind and Furi 1997; Jacob et al. 2002). Whether MHC-related odor preferences play a role in behaviors such as partner preference is difficult to investigate given the complexities of modern human society. However, fathers, grandmothers, and aunts have been reported to successfully identify the odor of a related infant without prior experience, which could point to a role in parental or nepotistic behavior of these learned odor signatures (Porter et al. 1986).

## 6.8 CONCLUDING REMARKS

Our understanding of the important influence of pheromones on mammalian behavior has advanced dramatically in the 50 years since the term was first proposed. These invisible chemical signals can elicit equally dramatic behavioral responses in mammals to those seen in insects. However, our understanding is fragmentary, with few examples in which the pheromonal signal, the sensory receptors on which it acts, and the behavioral response elicited have all been identified. The major advances in recent years have been based mainly on a single species—the mouse. Genetic technologies have revealed a surprisingly large repertoire of chemosensory receptors in mice that potentially detect pheromones. However, our knowledge of their natural ligands and behavioral role is limited by our lack of understanding of the natural behavior of mice and by the artificial laboratory environment in which they are studied.

Pheromones and the effects that they mediate are, by their nature, species-specific and may not be found in even closely related species. For example, the diversity of MUPs found in the house mouse, *Mus musculus*, appears to be a relatively recent evolutionary adaptation to its commensural lifestyle and is not observed in a closely related species of aboriginal mouse, *M. macedonicus*, which live at lower population densities (Robertson et al. 2007). Nevertheless, the genetic approaches used in mice, coupled with genomic analysis, provide a much needed focus for where and how to look for pheromonal signaling systems in other species.

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# 7 Odorant Receptors

Bettina Malnic, Daniela C. Gonzalez-Kristeller, and Luciana M. Gutiyama

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# 7.1 THE IDENTIFICATION OF ODORANT RECEPTORS (ORs)

The receptors responsible for odorant discrimination were first cloned in 1991 by Linda Buck and Richard Axel (Buck and Axel 1991). A series of physiological and biochemical experiments performed during the mid-1980s indicated that odorant activation of olfactory sensory neurons was mediated by a G-protein-dependent pathway, which led to activation of adenylyl cyclase, increases in intracellular concentrations of cyclic adenosine monophosphate (cAMP), activation of cyclic nucleotide-gated channels, and neuron depolarization (Firestein et al. 1991; Lowe et al. 1989; Nakamura and Gold 1987; Pace et al. 1985; Sklar et al. 1986; see also Chapter 8). The subsequent cloning of olfactory-specific genes coding for a G $\alpha$  protein (G $\alpha$ olf) (Jones and Reed 1989) and for a cAMP-gated channel (Dhallan et al. 1990) further strengthened the involvement of cAMP in odorant signal transduction. These experiments strongly indicated that the odorant receptors (ORs) should be G-protein-coupled receptors (GPCRs).

About the same time, the polymerase chain reaction (PCR) technique was developed (Saiki et al. 1988), and the first GPCRs had been identified. Comparison between the sequences of rhodopsin and  $\beta$ -adrenergic receptors indicated that receptors that couple to G-proteins showed related structures, with seven membrane-spanning regions (Dixon et al. 1986). Comparison of the sequences of a higher number of GPCRs (around 20 G-protein-compled receptor (GPCR) sequences were known by 1989) revealed that they all shared a related seven-transmembrane structure and they also shared

limited sequence motifs. In 1989, it was shown for the first time that degenerate primers could be used in PCR reactions to identify new members of the GPCR family (Libert et al. 1989).

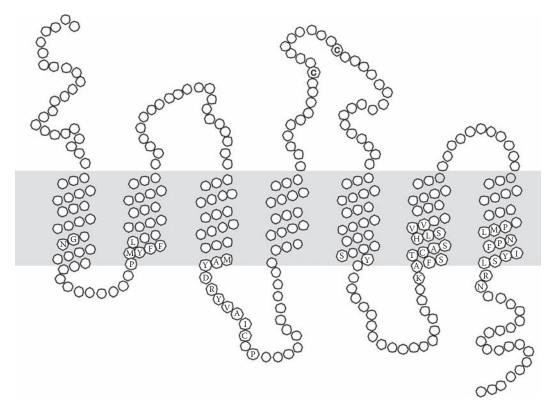
The approach used by Buck and Axel to isolate the Odorant receptor (OR) genes was based on the assumptions that the receptors should belong to a large family of GPCRs and their expression should be restricted to the olfactory epithelium (Buck and Axel 1991). Eleven degenerate primers that would allow amplification of all known GPCRs at the time were designed and all possible combinations were used in PCR reactions with rat olfactory epithelium cDNA. As a result, 64 bands of appropriate sizes were obtained in agarose gels. The next step was to screen these bands for the ones containing the OR genes. It was reasoned that if one of the bands contained cDNAs corresponding to multiple OR genes, four-cutter restriction enzymes would cleave the DNA into smaller fragments showing sizes that, when summed up, would produce a size greater than that of the original band. The 64 PCR bands were treated with the *Hinf*I or HaeIII restriction enzymes, and for most of the bands, restriction digestion generated fragments with sizes that summed up to the original band size. Therefore, these PCR products contained a single DNA species. However, restriction digestion of one of the bands produced fragments with sizes that summed to a value far greater than that of the original PCR product. This PCR band contained a mixture of different DNA species, each of which was amplified by the same pair of degenerate primers. The band was cloned into plasmid, and individual recombinant plasmids were sequenced. All sequences were different, but they all showed a GPCR-like structure. Using Northern blot analysis, it was also demonstrated that these receptors are expressed in the olfactory epithelium, but not in a further eight tissues analyzed, including the brain, retina, and liver (Buck and Axel 1991). In addition, in order to estimate the approximate size of the OR gene family, rat genomic libraries were screened for OR genes using a mixture of the OR cDNAs as probes. It was estimated then that the rat haploid genome should contain at least 500-1000 OR genes (Buck and Axel 1991; Buck 1992).

Comparison of different rat OR amino acid sequences revealed that, even though they are extremely diverse, they share conserved motifs that are characteristic of the OR family, such as GN in transmembrane domain I, PMYF/LFL in transmembrane domain II, MAYDRYVAIC in transmembrane domain III, KAFSTCA/GSHLSVV in transmembrane domain 6, and PMLNPFIYSLRN in transmembrane domain VII (Buck and Axel 1991) (Figure 7.1). Additional members of the OR family were identified by using degenerate primers matching these OR motifs in PCR reactions with olfactory epithelium cDNA or genomic DNA (since the OR coding region is contained in one single exon). Degenerate primers matching to the highly conserved motifs in transmembrane III and VI were very efficient in amplifying a large fraction of the mouse OR genes (Malnic et al. 1999; Michaloski et al. 2006; Ressler et al. 1993).

## 7.2 THE ODORANT RECEPTOR (OR) GENE FAMILY

#### 7.2.1 CLASS I AND CLASS II ODORANT RECEPTORS (ORS)

The ORs can be classified into two distinct classes, based on their amino acid sequences and phylogenetic distribution. The class I ORs were first identified in fish (Ngai et al. 1993) and in frog (Freitag et al. 1995), and it was later shown that teleost fish, including the goldfish, have only class I OR genes (Freitag et al. 1998). Semiaquatic animals, such as frogs, have both class I and class II OR genes (see also Chapter 4), and initially it was believed that mammals lacked functional class I ORs, and only contained class II ORs (Freitag et al. 1998). These findings suggested that the class I ORs (also denominated fishlike ORs) must be specialized in recognizing water-soluble odorants, while class II ORs (mammalianlike ORs) must recognize volatile odorants. However, recent analysis of genome sequences has shown that there are relatively large numbers of class I ORs in the genomes of human (Glusman et al. 2001; Malnic et al. 2004; Zozulya et al. 2001), mouse (Godfrey et al. 2004; Young et al. 2002; Zhang and Firestein 2002),



**FIGURE 7.1** Typical structure of an odorant receptor. The diagram illustrates one odorant receptor in the plasmatic membrane (shown in gray), with its seven putative transmembrane domains. Amino acids that are highly conserved among the majority of the OR proteins are shown. The remaining residues are extremely variable, consistent with the ability of the OR family to interact with a large number of odorants.

and other mammalian species (Niimura and Nei 2007). Even though the majority of OR genes belong to class II, between 10% and 20% of the ORs in mammals are class I ORs (Glusman et al. 2001; Niimura and Nei 2007; Zhang and Firestein 2002), indicating that class I ORs may also have important roles in mammalian olfaction.

# 7.2.2 THE SIZE OF THE ODORANT RECEPTOR (OR) GENE FAMILY

The recent availability of the complete genome sequences for several different species allows for the rapid identification of their OR genes. The fact that OR genes have intronless coding regions facilitates their identification. Typically, conserved amino acid sequences corresponding to known OR genes can be used as queries in TBLASTN searches of the genome sequences, to obtain new sequences that are related to OR genes (Glusman et al. 2001; Godfrey et al. 2004; Malnic et al. 2004; Niimura and Nei 2003; Zozulya et al. 2001). The retrieved nucleotide sequences are then translated into amino acid sequences and analyzed. A protein is considered an OR if it is encoded by a coding region of around 1 kb and if it contains the OR sequence motifs (or its variants) located at the appropriate positions (Figure 7.1).

In this way, the complete repertoires of OR genes have been identified for a large number of species. The OR repertoires vary in size and probably reflect the specific olfactory requirements of each one of these species. Some species have high numbers of intact (and potentially functional) OR genes, such as mouse (~1000), dog (~800), and opossum (~1200), while others have comparatively lower numbers of intact OR genes, such as human (~370), chimpanzee

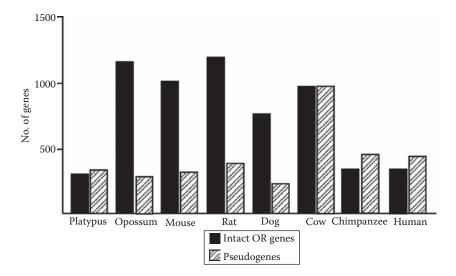


FIGURE 7.2 The size of the OR gene family in different mammalian species. The numbers of intact OR genes and pseudogenes were determined from platypus (*Ornithorhynchus anatinus*) (Niimura, Y. and Nei, M. 2007; Warren, W. et al. 2008), opossum (*Monodelphis domestica*) (Niimura, Y. and Nei, M. 2007), mouse (*Mus musculus*) (Godfrey, P.A., Malnic, B., and Buck, L.B. 2004: Niimura, Y., and Nei, M. 2005; Young, J.M. et al. 2002; Zhang, X. et al. 2004), rat (*Rattus norvegicus*) (Niimura, Y. and Nei, M. 2007; Quignon, P. et al. 2005), dog (*Canis familiaris*) (Niimura, Y. and Nei, M. 2007; Olender, T. et al. 2004; Quignon, P. et al. 2005), cow (*Bos taurus*) (Niimura, Y. and Nei, M. 2007), chimpanzee (*Pan troglodytes*) (Gilad, Y., Man, O., and Glusman, G. 2005; Go, Y., and Niimura, Y. 2008), human (*Homo sapiens*) (Glusman, G. et al. 2001; Malnic, B. Godfrey, P.A., and Buck, L.B. 2004; Niimura, Y., and Nei, M. 2003; Zozulya, S., Echeverri, F., and Nguyen, T. 2001).

(~370), and platypus (~300) (Figure 7.2). The numbers of pseudogenes, which do not express functional ORs, also vary among species, although not as dramatically: while humans and chimpanzees have around 460–480 pseudogenes, dogs, mice, and opossum have around 250–330 pseudogenes (Figure 7.2). The OR gene repertoires also reflect the habitats of the different species. It has been shown, for example, that marine mammals, which evolved from terrestrial ancestors and have adapted to the aquatic environment, have higher numbers of pseudogenes: in dwarf sperm whales and minke whales 77 and 58% of the OR genes are pseudogenes (Kishida et al. 2007). Dolphins completely lack class I OR genes, and their class II OR genes are all pseudogenes (Freitag et al. 1998).

The number of intact OR genes does not always correlate well with the olfactory abilities of a given species, indicating that other factors must also be involved. For example, dogs, which have a rich sense of smell, have a smaller number of OR genes than mice or rats (Figure 7.2), however, it is well known that they have larger surfaces of olfactory epithelia. Even though it is generally believed that primates have a poor sense of smell, behavioral studies have shown that primates, including humans, have a surprisingly good sense of smell (Laska et al. 2000). Humans have a smaller number of intact OR genes when compared to dogs or rodents (Figure 7.2). Interestingly, it was recently demonstrated that humans have an unexpectedly high number of glomeruli in their olfactory bulbs: while mice, which have around 1000 intact OR genes, have around 1800 glomeruli, humans, who have around 400 intact OR genes, have on average 5500 glomeruli per olfactory bulb (Maresh et al. 2008). Combined with the fact that the regions in the human brain that are involved

in olfactory processing are expanded when compared to other species, these anatomical differences may explain why humans have a good olfactory sensitivity despite having a small repertoire of OR genes (Shepherd 2004).

The expression in the olfactory epithelium has been confirmed for around 400 mouse OR genes through the screening of an olfactory cDNA library with degenerate olfactory receptor probes (Young et al. 2003). Using quantitative RT-PCR, it was also demonstrated that some OR genes are expressed at higher levels than others. It was observed that the expression levels can vary by 10- to 300-fold between genes. These same differences were found in three different mice that were examined, although there was a variation in the expression level of some OR genes between mice (Young et al. 2003). Differences may be due to increased numbers of expressing neurons, or to increased levels of OR gene transcripts per expressing neuron.

A high-throughput microarray analysis detected the specific expression of ~800 mouse OR genes in the olfactory epithelium (Zhang et al. 2004). Very few OR genes were expressed in the nonolfactory tissues that were analyzed, such as testis, liver, heart, cerebellum, and muscle, showing that although there might be a small number of OR genes expressed in other tissues, very few are exclusively expressed in nonolfactory epithelium tissues. Microarray analysis was also used to analyze the expression of human OR genes (Zhang et al. 2007). This study detected the expression of 437 OR genes, including pseudogenes, in the human olfactory epithelium.

# 7.2.3 COMPARATIVE GENOMICS OF ODORANT RECEPTORS (ORs)

Analysis of the composition of the OR gene families in different species has revealed several interesting points regarding the olfactory sensory function in these animals. In one study, a random group of 221 ORs was cloned from 10 different primate species, from prosimian lemur to human. Analysis of these OR gene sequences showed that the percentage of functional OR genes decreases, from New World monkeys to hominoids: while New World monkeys (like the squirrel monkey and marmoset) lack pseudogenes, Old World monkeys (macaque and baboon) have around 27% pseudogenes, and hominoids (chimpanzee, gorilla, orangutan, and human) have around 50% pseudogenes (Rouquier et al. 2000). These numbers may reflect the evolution of the olfactory sensory function in primates, which shows reduced olfactory abilities, when compared to other species, such as rodents and dogs. The recent availability of the complete sequence of the chimpanzee genome allowed for the comparison between the entire human and chimpanzee OR gene repertoires (Gilad et al. 2005; Gimelbrant et al. 2004; Go and Niimura 2008). While one study showed that humans have a significantly higher percentage of pseudogenes than chimpanzees (Gilad et al. 2005), another study, where an updated version of the chimpanzee genome sequence was analyzed, showed that the numbers of pseudogenes and intact OR genes are approximately the same between the two species (Go and Niimura 2008). However, this same study showed that 25% of the intact ORs are nonorthologous between human and chimpanzees (Go and Niimura 2008), indicating that the OR repertoires of these two species are somewhat different. Differences in OR repertoire composition may be responsible for species-specific abilities of odorant detection, and have also been observed when other species are compared. For instance, comparison of the human and mouse genome sequences identified 29 human ORs that have no counterpart in the mouse, and 177 mouse ORs with no counterpart in humans (Godfrey et al. 2004). Comparison of the canine and human OR genes showed that the canine repertoire has expanded relative to that of humans, leading to the emergence of specific canine OR genes (Quignon et al. 2003). For the class I ORs, no human or dog OR genes appeared to be species-specific, but for class II, one group of 26 ORs was considered to be dog-specific, as no counterparts were found in humans (Quignon et al. 2003).

## 7.2.4 CHROMOSOMAL DISTRIBUTION OF THE ODORANT RECEPTOR (OR) GENES

Early analysis of the chromosomal distribution of the mouse OR genes revealed that they are broadly distributed in the genome (Sullivan et al. 1996). In these experiments, the chromosomal locations of 21 mouse OR genes were experimentally determined using genetic crosses, and it was shown that these OR genes are clustered within multiple *loci* located in seven different chromosomes. Another study used fluorescence in situ hybridization (FISH) and fluorescence-activated cell sorter (FACS) experiments to determine the genomic locations of a large number of human OR genes (Rouquier et al. 1998). Degenerate oligonucleotide primers matching conserved sequences in OR genes were used to amplify OR genes directly from chromosomes, except for chromosomes 20 and X (Rouquier et al. 1998).

The chromosomal locations of the complete set of OR genes in different species have now been determined using bioinformatics analysis of the genomic sequences. The human OR genes are distributed in clusters located in all chromosomes, except for chromosomes 20 and Y (Glusman et al. 2001; Malnic et al. 2004; Niimura and Nei 2003). Similarly, the mouse OR genes are distributed in several *loci* located in all chromosomes, except for chromosomes 18 and Y (Niimura and Nei 2005; Zhang et al. 2004). Although the majority of the OR genes are concentrated in clusters, a few solitary OR genes have also been identified (Godfrey et al. 2004; Malnic et al. 2004).

## 7.3 EXPRESSION OF THE ODORANT RECEPTOR (OR) GENES

Even though the OR genes are broadly distributed all over the genome, their expression is tightly regulated by a still undeciphered mechanism. Basically, there are three levels of OR gene expression. First, even though OR gene expression was reported in nonolfactory tissues, principally in the testis (Mombaerts 1999; Parmentier et al. 1992; Spehr et al. 2003), the vast majority of the OR genes are exclusively expressed in the olfactory epithelium (Zhang et al. 2004). Second, each OR gene is expressed in only one out of four OR expression zones in the olfactory epithelium. Third, each olfactory sensory neuron expresses one single OR gene allele, while the other genes remain silent.

#### 7.3.1 ZONAL EXPRESSION OF ODORANT RECEPTORS (ORS) IN THE OLFACTORY EPITHELIUM

A series of in situ hybridization experiments using ORs as molecular probes showed that the olfactory epithelium is divided into four distinct spatial zones in which different groups of OR genes are expressed (Ressler et al. 1993; Vassar et al. 1993). The zones are symmetrically distributed along the dorsal–ventral axis of the epithelium, with zone 1 localized in the dorsal region and zone 4 in the ventral region of the epithelium (according to the nomenclature of Sullivan et al. [1996]). Each zone is likely to express hundreds of OR genes, and the olfactory sensory neurons that express a given receptor are randomly dispersed within its expression zone. The class I OR genes are mostly expressed in zone 1 (Tsuboi et al. 2006; Zhang et al. 2004). Some class II OR genes are also expressed in zone 1, but the majority is expressed in zones 2–4 (Miyamichi et al. 2005; Zhang et al. 2004).

In another study, 80 class II OR genes were analyzed for their expression pattern in the olfactory epithelium (Miyamichi et al. 2005). This analysis showed that, with the exception of the zone 1 OR genes, the OR genes did not fit in one of the previously described four expression zones, but their expression areas are distributed in an overlapping and continuous manner along the dorsal–ventral axis of the olfactory epithelium, such that no clear borders are present between the neighboring zones.

It has been expected that each zone in the olfactory epithelium should express about one-quarter of the OR gene repertoire. The spatial distribution of OR gene expression in the olfactory epithelium was analyzed using a high-throughput microarray analysis (Zhang et al. 2004). Regions corresponding to zone 1 and zones 2–4 were microdissected from the olfactory epithelium and tested on the microarray for OR gene expression. Interestingly, zone 1 contained more than one-third of all OR genes expressed in the olfactory epithelium. Together with the fact that class I ORs are specifically expressed in zone 1, these results indicate that zones 1 and 2–4 may play distinct roles in olfaction.

The functional implications of the zonal organization of OR gene expression in the olfactory epithelium are still unclear. The axonal projection from the olfactory epithelium to the olfactory bulb is also organized along the dorsal-ventral axis, that is, zone 1 neurons project their axons to the dorsal region of the bulb, while zone 4 neurons project their axons to the ventral region of the bulb. This pattern of projection suggests that the zonal segregation of ORs, and consequently of the sensory information, in the nasal cavity is maintained in the olfactory bulb. However, it is not yet known whether ORs in different zones respond to different classes of odorants.

#### 7.3.2 ONE OLFACTORY SENSORY NEURON ONE ODORANT RECEPTOR AND (OR)

Olfactory sensory neurons select, from over a thousand possible choices, one single OR gene allele to express (Chess et al. 1994; Malnic et al. 1999; Ressler et al. 1993; Serizawa et al. 2000; Vassar et al. 1993). Axons of neurons that express one same given OR converge onto two or a few glomeruli at two specific sites in the olfactory bulb (Ressler et al. 1994; Vassar et al. 1994). Interestingly, specific glomeruli show approximately the same locations in different individuals. These results indicate that the information provided by different ORs in the nose is organized into a stereotyped sensory map in the olfactory bulb.

The receptor type that is chosen will determine the range of odorants to which this neuron will respond, and it has been shown that it is also required for axonal targeting to specific glomeruli in the olfactory bulb (Mombaerts et al. 1996; Wang et al. 1998). OR gene choice is, therefore, fundamental for the functional organization of the olfactory system.

Different mechanisms have been proposed for the control of OR gene expression. One possibility considered was that OR gene choice could be controlled by specific DNA rearrangements in the olfactory neurons (Kratz et al. 2002). However, it was demonstrated that mice cloned from olfactory sensory nuclei, despite having originated from a neuron expressing a single OR type, showed no irreversible DNA changes in the OR genes and exhibited a normal range of OR gene expression (Eggan et al. 2004; Li et al. 2004). It has also been considered that each OR gene could be selected by a unique combination of transcription factors. However, the fact that OR transgenes and their corresponding endogenous OR genes are not coexpressed in the same neuron (Serizawa et al. 2000) argues against this possibility.

It has been demonstrated that the monoallelic expression of an OR gene is regulated by a negative feedback mechanism that requires a functional OR protein (Lewcock and Reed 2004; Serizawa et al. 2003). In addition, it was shown that immature olfactory neurons expressing a given OR can switch receptor expression at a low frequency, while neurons expressing a mutant (nonfunctional) OR can switch expression with a greater probability (Shykind et al. 2004). These results indicate that after an OR gene is stochastically selected for expression by a limiting factor, its corresponding OR protein product mediates a feedback signal that results in the maintenance of the receptor choice (Serizawa et al. 2004; Shykind 2005).

Little is known about the role of *cis*-regulatory sequences in the regulation of OR gene expression. In studies using transgenic mice, different sizes of genomic DNA segments containing OR genes were tested for their ability to drive an OR expression similar to that of the endogenous gene. It was demonstrated that short pieces of DNA located upstream of the coding region, ranging from 460 to 6.7 kb, are sufficient for expression of the ORs M4, M71, and MOR23 (Qasba and Reed 1998; Vassalli et al. 2002). However, large segments of around 200 kb are required to obtain expression of MOR28 (Serizawa et al. 2000). Sequence comparison of the mouse and human genome revealed a 2 kb conserved sequence located ~75 kb upstream of the MOR28 cluster. This region,

denominated H region or H enhancer, was proposed to work as a *cis*-acting locus control region (LCR), which would activate the expression of one single OR gene member from within the MOR28 cluster (Serizawa et al. 2003).

A detailed analysis of the minimal proximal promoter of OR M71 showed that it contains homeodomain and O/E-like binding sites (Nishizumi et al. 2007; Rothman et al. 2005). Mutations in these binding sites abolish its ability to drive OR gene expression in transgenic animals, indicating that homeodomain and *olf-1* (O/E-like) transcription factors are involved in OR gene expression. Consistent with this finding, homeodomain and O/E-like binding sites have been identified in a large number of OR gene promoters (Hoppe et al. 2006; Michaloski et al. 2006). O/E-like binding sites were also identified in the promoters of several other olfactory specific genes, such as Golf, adenylyl cyclase III (ACIII), olfactory cyclic nucleotide-gated channel (OcNC), and olfactory marker protein (OMP) (Kudrycki et al. 1993; Wang et al. 1993). Interestingly, the H region also contains homeodomain and O/E-like binding sites (Hirota and Mombaerts 2004), and it was shown that mutations in these sites abolish the ability of the H region to drive expression of OR genes in transgenic animals (Nishizumi et al. 2007).

So far, two different homeodomain transcription factors have been implicated in OR gene expression. Lhx2, a LIM-homeodomain protein, was shown to bind to the MOR71 promoter region (Hirota and Mombaerts 2004). Lhx2-deficient mice lack mature olfactory sensory neurons, indicating that this homeodomain protein is required for olfactory sensory neuron development (Hirota and Mombaerts 2004; Kolterud et al. 2004). In these mutant mice, the expression of class II OR genes is abolished, while most class I OR genes are still expressed in a few OMP-positive neurons located in the dorsal region (corresponding to zone 1) of the olfactory epithelium (Hirota et al. 2007). These results indicate that Lhx2 is directly involved in class II OR gene expression, but is not required for class I OR gene expression. The results also suggest that class I and class II OR gene expression is regulated by distinct mechanisms.

Recently, the Emx2 homeobox transcription factor has also been implicated in OR gene regulation (McIntyre et al. 2008). Emx2 was shown to bind to the mouse OR71 gene promoter (Hirota and Mombaerts 2004) and to be expressed in the olfactory epithelium (Nedelec et al. 2004). Emx2mutant mice develop a normal olfactory epithelium, except that they have a reduced number of mature olfactory sensory neurons (McIntyre et al. 2008). The expression of many OR genes is reduced greater than the 42% reduction in mature olfactory sensory neurons, indicating that the absence of Emx2 is not altering OR gene expression only because of a general defect in olfactory sensory neuron development. Altogether, these results indicate that Emx2 acts directly on OR gene promoters to regulate gene transcription. Interestingly, a few OR genes show increased expression, when compared to wildtype mice (McIntyre et al. 2008). It is possible that these OR genes do not depend on Emx2 to be transcribed.

The *olf1* (O/E) transcription factors are specifically expressed in the olfactory neurons and in B-lymphocytes (Hagman et al. 1993; Wang and Reed 1993). The roles of O/E-like proteins in OR gene expression are still unclear. Disruption of *olf-1*-like genes does not alter OR gene expression (Lin and Grosschedl 1995; Wang et al. 2003), possibly due to the functional redundancy of the multiple O/E family members expressed in the olfactory epithelium (O/E1, O/E2, O/E3, and O/E4; Wang et al. 1997, 2002). However, it was demonstrated that O/E2- and O/E3-mutant mice show defects in the projection of olfactory neurons to the olfactory bulb, indicating that the O/E genes function may not be completely redundant (Wang et al. 2003).

Experiments using the chromosome conformation capture (3C) technique showed that in the nuclei of olfactory sensory neurons, the H region, which is located on chromosome 14, associates with OR gene promoters located in different chromosomes (Lomvardas et al. 2006). DNA and RNA FISH analysis demonstrated that the H region is associated with the single OR gene that is transcribed in a given neuron. Also, in the olfactory sensory neurons, one of the two H alleles is methylated, and therefore inactive. Based on these results, a model for OR gene choice was proposed, where one single trans-acting H enhancer element allows stochastic activation of one

single OR gene allele per olfactory sensory neuron. However, it was subsequently shown that mice that have the H region deleted show regular expression of OR genes, except for some of the OR genes that are located within the MOR28 cluster. In this case, the expression of the three OR genes located proximal to the H region, MOR28, MOR10, and MOR83, was abolished (Fuss et al. 2007; Nishizumi et al. 2007). These results indicate that the H region acts in *cis* to promote expression of these three genes, but is not an essential trans-acting enhancer that regulates monoallelic expression of OR genes in olfactory sensory neurons.

In order to obtain expression of a particular OR gene in a large number of olfactory sensory neurons, transgenic mice were constructed, where the full length of the OR coding sequence is placed under the control of the promoter of genes that are abundantly expressed in these neurons, such as the OMP or G $\gamma$ 8 genes (Nguyen et al. 2007). However, these constructs did not result in transgenic expression of the OR gene. When the OR coding sequence is replaced by a different unrelated GPCR, like the human taste receptor hT2R16 or the opioid receptor RASSL, these GPCRs are expressed in the vast majority of the OMP or G $\gamma$ 8 positive neurons, indicating that the suppression of OR gene expression in the olfactory sensory neurons is not extended to GPCRs in general, but is specific to OR. In addition, when the OR coding sequence, a motif known to be essential for G-protein activation and signal transduction, the OR gene expression is still suppressed, showing that OR function is not required for OR silencing. These results are consistent with other experiments that showed that the mechanism of negative feedback regulation may not require G-protein-mediated signaling (Imai et al. 2006).

The inhibition of the OR transgene expression could be part of the normal process that controls endogenous OR gene expression, so that one single type of OR gene is expressed, while the remaining OR genes are repressed. Interestingly, OR gene expression was achieved only when the OR coding sequence was separated from the promoter sequences: for example, a transgenic line where the OMP (or G $\gamma$ 8) promoter sequence drives the expression of the tetracycline transactivator is crossed with a transgenic mouse carrying a TetO promoter driving the expression of the OR gene (Nguyen et al. 2007). These results suggest that both the OR coding sequence and the promoter driving its expression, must be involved in OR gene regulation.

# 7.4 ODORANT SIGNAL TRANSDUCTION THROUGH ODORANT RECEPTORS (ORs)

Antibodies recognizing distinct ORs have been used to determine their cellular distribution. These experiments showed that the receptor proteins are localized in the cilia of olfactory sensory neurons, the site of odorant signal transduction (Barnea et al. 2004; Menco et al. 1997; Schwarzenbacher et al. 2005; Strotmann et al. 2004). Odorant signal transduction is initiated by the binding of odorants to ORs and the activation of the associated heterotrimeric G-protein, Golf. Once activated, G $\alpha$ olf exchanges guanosine diphosphate (GDP) for guanosine triphosphate (GTP), the GTP-bound G $\alpha$ olf subunit dissociates from the G $\beta/\gamma$  complex and activates ACIII, leading to increased intracellular levels of cAMP and opening of cyclic nucleotide-gated channels. The resulting influx of Na<sup>+</sup> and Ca<sup>2+</sup> ions ultimately leads to the generation of an action potential in the olfactory neuron axon (Firestein 2001; Mombaerts 2004; Ronnett and Moon 2002; see also Chapter 8).

Initially, it was believed that two separate types of intracellular signaling pathways could be activated by different classes of odorants: the cAMP pathway and the IP3 pathway (Boekhoff et al. 1990; Huque and Bruch 1986; Ronnett et al. 1993; Sklar et al. 1986). However, because mice that are knockout for components of the cAMP pathway do not respond to odorants of any class (Belluscio et al. 1998; Brunet et al. 1996; Wong et al. 2000), it is believed that olfactory transduction is exclusively mediated by the cAMP pathway, although it is possible that the IP3 pathway plays a modulatory role (Spehr et al. 2002), or is involved in signaling in different types of cells in the olfactory epithelium (Elsaesser et al. 2005; Gold 1999; Liberles and Buck 2006; Lin et al. 2007; see also Chapter 9).

Recently, additional proteins that are likely to be involved in OR function have been identified. The receptor transporting proteins, RTP1, RTP2, and REEP1, which are specifically expressed in the olfactory sensory neurons in the olfactory epithelium, were shown to associate with ORs when coexpressed in HEK293T cells (Saito et al. 2004). It was also shown that they promote cell surface expression of ORs in HEK293T cells. In vivo, it is possible that they work as chaperones that aid in OR folding and/or trafficking to the plasma membrane (Saito et al. 2004).

The guanine nucleotide exchange factor (GEF) Ric-8B, interacts with G $\alpha$ olf (Von Dannecker et al. 2005). Ric-8B is specifically expressed in olfactory sensory neurons and in a few regions in the brain where G $\alpha$ olf is also expressed, such as the striatum, nucleus accumbens, and olfactory tubercle (Von Dannecker et al. 2005). Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP to generate an activated form of G $\alpha$ , which is then able to activate a variety of effectors. Consistent with this potential function, Ric-8B is able to amplify dopamine receptor and OR signaling through G $\alpha$ olf (Von Dannecker et al. 2005, 2006). It was recently demonstrated that Ric-8B, besides interacting with G $\alpha$ olf, also interacts with G $\gamma$ 13, which is also expressed in mature olfactory sensory neurons (Kerr et al. 2008). In addition, it was shown that G $\beta$ 1 is the G $\beta$  subunit that is predominantly expressed in the mature olfactory sensory neurons and that the G $\beta$ 1 protein is localized to the cilia of olfactory sensory neurons, together with G $\gamma$ 13 and Ric-8B (Kerr et al. 2008).

The physiological roles of the RTPs and Ric-8B in OR function should be clarified through the analysis of mice that are knockout for these proteins.

# 7.5 ODORANT RECEPTORS (ORs) AND AXONAL TARGETING IN THE OLFACTORY BULB

The experiments using antibodies against ORs showed that the receptors are also localized in the axonal processes of the olfactory sensory neurons (Barnea et al. 2004; Menco et al. 1997; Schwarzenbacher et al. 2005; Strotmann et al. 2004), consistent with the role they play in axonal targeting to specific glomeruli in the olfactory bulb (Feinstein et al. 2004; Wang et al. 1998). The mechanisms through which the ORs regulate axonal projection are not completely understood. ORs in the axonal terminals could recognize guidance molecules in the olfactory bulb to form specific glomeruli (Mombaerts 2006), or, alternatively, axons could coalesce into a glomerulus independently of the presence of a target in the bulb, but as a consequence of homophilic interactions between axons containing the same OR type (Feinstein et al. 2004; Feinstein and Mombaerts 2004). Recent studies have demonstrated that OR-derived cAMP signals are essential for axonal targeting in the bulb (Imai et al. 2006; Serizawa et al. 2006). In this model, each OR type generates a unique level of cAMP. The levels of cAMP define the expression levels of guidance molecules, which determine the anterior-posterior topography of axonal projection in the olfactory bulb. It is not yet clear whether ORs present in the cilia or in the axonal terminals, or both, are involved in the generation of the cAMP signals that regulate the formation of the OR-specific glomerular map.

## 7.6 ODORANT DISCRIMINATION BY ODORANT RECEPTORS (ORs)

#### 7.6.1 COMBINATORIAL RECEPTOR CODES FOR ODORANTS

Even though mammals have only 100s of functional ORs, they can discriminate a much higher number (several thousands) of odorants. In order to understand how the olfactory system utilizes the OR gene family to discriminate odorants, one should determine the odorant specificities of individual ORs. However, to date, only a few ORs have been linked to odorants they recognize because ORs cannot be efficiently expressed in heterologous cells (Malnic 2007). They are usually retained in the endoplasmatic reticulum and cannot reach the plasmatic membrane (Gimelbrant et al. 1999, 2001; Katada et al. 2004; Lu et al. 2003, 2004). In order to circumvent this problem, a combination of Ca<sup>2+</sup> imaging and single-cell RT-PCR was used to identify the ORs expressed by olfactory neurons that responded to different aliphatic odorants (Malnic et al. 1999), to lyral (Touhara et al. 1999), or to eugenol (Kajiya et al. 2001). In these experiments, dissociated olfactory sensory neurons are loaded with the Ca<sup>2+</sup> sensitive dye, fura-2, and exposed to a panel of odorants. The increases in Ca<sup>2+</sup> concentration are recorded as fluorescence decreases in the intensity of the emitted light (510 nm) of neurons excited at 380 nm (Malnic et al. 1999). The neurons that respond to the odorants are individually transferred to micro tubes and a two-step, single-cell RT-PCR/PCR procedure is used to identify the OR genes expressed by each neuron. In a primary PCR reaction, cDNAs derived from all of the mRNAs expressed by a neuron are amplified. In a secondary PCR reaction, the primary PCR products are used as template with degenerate primers that specifically amplify members of the OR family. In this way, we can identify the OR expressed by the recorded neuron.

These experiments showed that one OR can recognize multiple odorants, but that different odorants are recognized by different combinations of receptors (Malnic et al. 1999). Thus, the olfactory receptor family is used in a combinatorial manner to discriminate odorants. Given that there are around 1000 OR genes, this combinatorial receptor-coding scheme should permit the detection of a vast number of odorants. It should also permit the olfactory system to discriminate between odorants that have very similar structures, such as aliphatic odorants with different carbon chain lengths (Malnic et al. 1999). These results are consistent with previous observations that single olfactory sensory neurons (Firestein et al. 1993; Sato et al. 1994; Sicard and Holley 1984) and individual glomeruli in the olfactory bulb (Adrian 1950; Friedrich and Korsching 1997; Leveteau and MacLeod 1966; Mori et al. 1992) can be stimulated by multiple odorants.

#### 7.6.2 FUNCTIONAL EXPRESSION OF ODORANT RECEPTORS (ORs) IN HETEROLOGOUS CELLS

As explained above, it is believed that the major reason for the inefficient functional expression of ORs in heterologous cells is the fact that the receptors do not reach the plasma membrane. However, recent advances have improved the expression of ORs in heterologous cell lines. Some of the techniques being used to deorphanize ORs in heterologous cells are based on strategies that should contribute to increased amounts of receptors on the cell surface. It has been demonstrated that fusion of the 20 N-terminal amino acids of rhodopsin to the N-terminal region of ORs facilitates cell surface expression of at least some ORs (Krautwurst et al. 1998). Using cotransfection, ORs with an N-terminal segment of rhodopsin ("rho-tagged ORs") can be expressed in heterologous cells together with the  $G\alpha_{15/16}$  subunits, which can promiscuously couple receptors to the phospholipase C pathway (Krautwurst et al. 1998). Receptor activation by odorants results in increased intracellular Ca<sup>2+</sup>, which can be measured at the single-cell level using Ca<sup>2+</sup> sensitive dyes.

ORs expressed in heterologous cells can also couple to G $\alpha$ olf (the natural partner of ORs), leading to odorant-induced increases in cAMP (Kajiya et al. 2001; Shirokova et al. 2005). A cell line that stably expresses the olfactory signal transduction molecules G $\alpha$ olf and cyclic nucleotide-gated channel subunit A2 (CNGA2) (named HeLa/Olf cell line), has also been used to functionally express ORs (Shirokova et al. 2005). Importantly, it was observed that the use of nonolfactory G-proteins may alter the OR responses to particular odorants, indicating that heterologous systems that use endogenous olfactory transduction molecules are more likely to reproduce OR physiological responses (Krautwurst 2008; Shirokova et al. 2005).

It was also demonstrated that coexpression with the olfactory-specific RTPs in HEK293T cells promotes OR functional surface expression (Saito et al. 2004). The RTPs are transmembrane proteins and were shown to directly interact with ORs in coimmunoprecipitation assays (Saito et al. 2004). It was demonstrated that cotransfection of RTP1 and OR also enhances surface expression of RTP1; it is possible that they work as coreceptors with ORs. They could also be involved in different functions, such as OR folding, export from the endoplasmic reticulum, or vesicle transport (Saito et al. 2004). HEK293T cells stably expressing G $\alpha$ olf, RTP1, RTP2, and REEP1 were established (named Hana3A cell line) and can now be used to investigate the specificities of a large number of ORs (Saito et al. 2004).

In a different approach, it was demonstrated that coexpression with the GEF Ric-8B and G $\alpha$ olf results in functional expression of ORs in HEK293T cells (Von Dannecker et al. 2006). Importantly, it was shown that Ric-8B promotes functional expression of untagged (without a rho tag) ORs, which is advantageous because it is possible that receptor protein modifications interfere with the ligand affinities. GEFs are considered to work as positive regulators of GPCR signaling. Therefore, in this case, functional expression of ORs is not mediated by an increase in the amount of receptors on the cell surface, but instead, results from the amplification of the OR signaling through the G-protein.

Interestingly, it was recently demonstrated that the use of a combination of Ric-8B, RTP1S (a short form of RTP1), and rho tags results in an improved heterologous expression of ORs (Von Dannecker et al. 2006; Zhuang and Matsunami 2007). The use of these methods in the future should facilitate the deorphanization of mammalian ORs.

## 7.7 HUMAN ODORANT RECEPTORS (ORs)

The fact that almost half of the human ORs repertoire is apparently nonfunctional (Go and Niimura 2008; Rouquier and Giorgi 2007) suggests that during the process of evolution, olfaction may have lost importance for primates. Even though the number of functional OR genes is smaller when compared to other species, humans have a very sensitive sense of smell, which is important for the detection of odorants that are essential for life, such as the smell of smoke (detection of fire) and the smell of rotten food (to avoid its ingestion). Smells are also intimately related to how humans taste food (Shepherd 2004; see also Chapter 16).

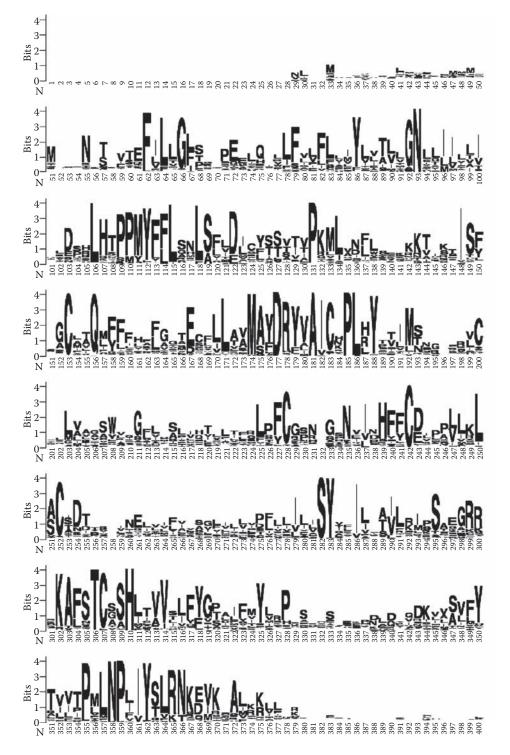
Comparison between the human and mice OR gene repertoires showed that, despite the smaller number of intact human ORs, the vast majority of human OR subfamilies have counterparts in the mouse repertoire (Godfrey et al. 2004). These results suggest, in principle, that the majority of odorant features detectable by one species may also be recognized by the other. However, mice may have a better ability to discriminate between similar odorants than humans (Godfrey et al. 2004).

#### 7.7.1 DEORPHANIZED HUMAN ODORANT RECEPTORS (ORS)

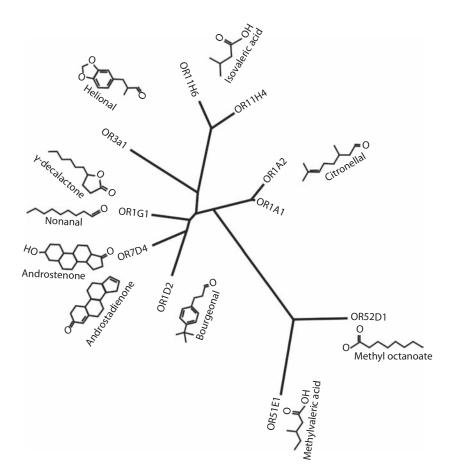
There are approximately 400 functional ORs in humans (Glusman et al. 2001; Malnic et al. 2004; Niimura and Nei 2003; Zozulya et al. 2001). Analysis of the amino acid sequences of all intact human ORs shows that they share the typical OR motifs (Figure 7.3). To date, only a few human ORs have been linked to odorants they recognize. Some examples are shown in Figure 7.4. Two of these (OR51E1 and OR52D1) are class I ORs, and the remaining are class II ORs. Several approaches, which use coexpression with different G $\alpha$  subunits, have been used to deorphanize these ORs. For instance, receptors OR1A1 and OR1A2 were functionally expressed in Hela/Olf cells (via G $\alpha$ olf) and can specifically detect citronellic terpenoid odorants (Schmiedeberg et al. 2007). In a previous study, Shirokova and colleagues demonstrated that the mouse orthologue, Olfr43, also responds to this agonist using the same system (Shirokova et al. 2005). Some human ORs were analyzed by using HEK293 cells that coexpress ORs and the promiscuous G $\alpha$ 16 subunit, which couples the receptor to the IP3-mediated pathway (Krautwurst et al. 1998). OR1G1 was shown to preferentially respond to 9-carbon aliphatic molecules, and can be inhibited by some odorants with structures that are related to that of the agonists (Sanz et al. 2005). OR52D1, a class I OR, was shown to respond to methyl octanoate (Sanz et al. 2005).

## 7.7.2 Odorant Receptors (ORs) and Pheromones

Pheromones are intraspecific chemical signals that regulate a series of innate behaviors, such as reproduction and aggression (Brennan and Zufall 2006; see also Chapter 6). The detection



**FIGURE 7.3** Motif patterns found in the human ORs. Weblogo representation for 397 human OR amino acid sequences. Sequences were aligned using Clustalw.



**FIGURE 7.4** Phylogenetic relationships of deorphanized human ORs and their ligands. OR1A1 and OR1A2 (Schmiedeberg, K. et al. 2007), OR1D2 (Spehr, M. et al. 2003), OR1G1 (Sanz, G. et al. 2005), OR3A1 (Jaquier, V. et al. 2006; Wetzel, C.H. et al. 1999), OR7D4 (Keller, A. et al. 2007), OR11H6 and OR11H4 (Menashe, I. et al. 2007), OR51E1 (Fujita, Y. et al. 2007), OR52D1 (Sanz, G. et al. 2005).

of pheromones is mediated by an accessory olfactory system, the vomeronasal system, which is anatomically segregated from the main olfactory system. Two different families of vomeronasal receptors, the V1Rs and V2Rs, which are also GPCRs, are expressed in the vomeronasal neurons and are responsible for the recognition of pheromones (Dulac and Torello 2003). Humans, however, do not have a functional vomeronasal organ (Brennan and Zufall 2006). In addition, the vast majority of human V1Rs and all V2Rs are pseudogenes (Young et al. 2005; Young and Trask 2007). Therefore, it is possible that the main olfactory system is the organ that detects pheromones in humans (Brennan and Zufall 2006).

The comparison between the OR repertoires in humans and mice has also revealed the presence of species-specific subfamilies of OR genes. These ORs are strong candidates to be involved in the detection of pheromones, or maybe of odorant classes that are detected by only one species (Godfrey et al. 2004).

The OR repertoires of humans and a closer species in terms of evolution to humans, the chimpanzee, were also compared (Gilad et al. 2005). Although their different habitats should result in different odorant detection needs, these species share the majority of OR genes. However, two subfamilies that are specific to chimpanzees and three subfamilies that are specific to humans were identified. The ORs that constitute these species-specific subfamilies show 99% amino acid sequence identity among themselves, with the exception of one human subfamily, which is composed of ORs with 70% identity among themselves (Gilad et al. 2005). Also, as described above, another study showed that 25% of the intact ORs are nonorthologous between humans and chimpanzees (Go and Niimura 2008). The agonists of species-specific ORs, which may have acquired species-specific functions, are still unknown, but their identification should be of interest.

A second family of GPCRs, known as trace amine-associate receptor (TAARs) and expressed in the olfactory epithelium, was recently described (Liberles and Buck 2006). The term "traceamines" refers to  $\beta$ -phenylethylamine, p-tyramine, tryptamine, and octopamine, which are present at very low concentrations (nanomolar range) in mammalian tissues. While mice have 15 TAARs, only 6 TAARS were identified in humans (Liberles and Buck 2006). These receptors were shown to bind to volatile amines found in urine, which are linked to stress or are differentially concentrated in male vs female urine, and therefore are believed to be involved in pheromone detection. The role of TAARs in humans remains unknown.

## 7.7.3 ODORANT RECEPTORS (ORS) AND PERCEPTION

An interesting feature of the human OR repertoire is that it is highly polymorphic. From pioneering perception studies, it is known that the ability to detect some odorants can vary greatly between individuals. Individuals that only detect some odorants when present in high concentrations or individuals that do not detect some odorants at all are relatively common in the human population (Amoore 1967, 1977; Amoore and Steinle 1991). Heterologous systems can now be used to functionally express polymorphic variants of human ORs to correlate differences in the structure of ORs in a population and their agonists.

A small number of polymorphisms in human OR genes have been described so far, but with the increasing availability of genomic sequences and single nucleotide polymorphisms (SNPs) from different individuals, new ones should be identified. For example, analysis of the 17 human OR genes present in the chromosomic region 17p13.3 revealed the existence of polymorphisms in the coding region of 14 of the OR genes, which show a total of 26 SNPs; from these, 21 are cSNPs (coding SNPs), that is, modifications that result in amino acid changes in the structure of the protein (Sharon et al. 2000).

In another study, 51 OR gene *loci* that are potential pseudogenes were analyzed in 189 individuals from several ethnic origins. The results revealed a high level of interindividual variability (Menashe et al. 2003). Interestingly, it was observed that non-African individuals had fewer functional OR genes than African American individuals. These results suggest that different evolutionary pressures may have shaped the OR repertoire in different human populations (Menashe et al. 2003).

Recently, Keller and colleagues showed that SNP variations in OR7D4 (Figure 7.4) correlate to differences in the perception of two substances that bind to this OR: androstenone and androstadienone (Keller et al. 2007). Individuals containing one or two nonfunctional alleles from gene OR7D4, that is, with two SNPs that result in two amino acid substitutions, are less sensitive to the abovementioned agonists. Another recent study identified a single SNP in the gene OR11H7P. This mutation in some individuals changes their sensitivity to the OR agonist, isovaleric acid (Menashe et al. 2007). These results support a relationship between genotypic and phenotypic variability in human olfaction.

Recent studies show that most of the human genome variation is not only due to SNPs, but also to structural variations of the genome, such as deletion of kilo- or megabase pairs, duplications, insertions, and inversions (Kidd et al. 2008; Korbel et al. 2007; Redon et al. 2006). Structural variations that affect the number of copies of a given region larger than 1 kb are called copy number variants

(CNVs) (Feuk et al. 2006). Recently, the impact of CNVs on the individual OR gene content has been analyzed. It was shown that ~30% of the human OR genes, including pseudogenes, are polymorphic with respect to copy number (Hasin et al. 2008; Nozawa et al. 2007; Young et al. 2008). Experimental validation of some CNV ORs in 50 individuals demonstrated that some ORs are deleted in some individuals and not in others, while others are duplicated in a subset of individuals (Young et al. 2008). The combination of SNPs and CNVs in the OR gene family among different individuals must have a significant impact on our olfactory abilities.

Recent work by Saito and colleagues identified agonists for 10 human and 56 mouse ORs by using a high-throughput screening(Saito, H., Chi, Q., Zhuang, H., Matsunami, H. and Mainland, J.D. (2009) Odor coding by a Mammalian receptor repertoire. *Sci. Signal.* 2(60): ra9).

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# 8 Signal Transduction in Vertebrate Olfactory Cilia

Simone Pifferi, Anna Menini, and Takashi Kurahashi

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# 8.1 INTRODUCTION

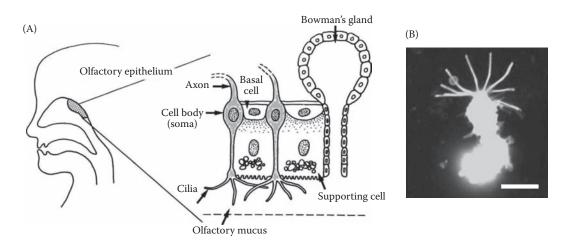
The initial steps of olfaction occur in primary sensory neurons located in the olfactory epithelium of the nasal cavity of vertebrates. These neurons are responsible for the detection of odorant molecules present in the surrounding environment and the generation of the neural signal that is transmitted to the brain. The morphology of the primary sensory neurons was described by Max Schultze in the second half of the nineteenth century (for review, see Zippel 1993), but only about 100 years later the first reviews describing some functional properties of these neurons were published (Getchell 1986; Lancet 1986). Primary sensory neurons of the olfactory epithelium, often indicated by various names: olfactory receptor cells (ORCs), olfactory sensory neurons (OSNs), or olfactory receptor neurons (ORNs), are bipolar neurons with a single dendrite that terminates with a knob, from which several tiny cilia protrude, where the

transduction of the olfactory signal takes place. Odorant molecules bind to odorant receptors, and this interaction triggers an increase in the intraciliary concentration of cyclic adenosine monophosphate (cAMP) through the activation of the receptor-coupled G-protein and adenylyl cyclase (AC). Cyclic nucleotide-gated (CNG) channels located in the ciliary membrane are directly activated by cytoplasmic cAMP, causing a depolarizing influx of Na<sup>+</sup> and Ca<sup>+</sup> ions. The odorant-induced inward transduction current has been shown to be composed not only of a cation influx through CNG channels, but also of a Cl<sup>-</sup> efflux through Cl<sup>-</sup> channels activated by Ca<sup>2+</sup> (Cl<sub>(Ca)</sub> channels). This chapter will review the molecular mechanisms underlying the functional role of vertebrate olfactory cilia.

# 8.2 ANATOMICAL GEOMETRY OF THE VERTEBRATE OLFACTORY RECEPTOR CELL (ORC) AND SENSORY CILIA

In the nose of vertebrates, odorants are detected by the main olfactory epithelium located in the nasal cavity (Figure 8.1A). The main olfactory epithelium is composed of several types of populations of sensory cells. The reader is referred to Chapter 9 of this book for a detailed discussion of subsystems of sensory cells (see also Tirindelli et al. 2009). The three main types of cells are the principal ORCs, the supporting cells, and the basal cells. ORCs are bipolar neurons with a single dendrite from which tens of cilia elongate into the olfactory mucus that covers the surface of the epithelium (Menco and Morrison 2003), and a single axon that projects directly to the olfactory bulb of the brain.

At the apical part of the dendrite, ORCs form tight junctions with the neighboring cells, mainly with supporting cells. Therefore, ORCs are in contact with different subregions defined by the surrounding environments. The apical region faces the outside of the body and is directly exposed to odorants, while the other parts of the neuron are embedded in the epithelium and are surrounded by the interstitial solution. Since the former is directly exposed to the external



**FIGURE 8.1** (See color insert following page 206.) Olfactory epithelium and olfactory receptor cell. (A) Schematic diagram of the localization of the human olfactory epithelium in the upper part of the nasal cavity (left panel). Schematic diagram showing the histological organization of the olfactory epithelium. Olfactory receptor cells (ORCs) are in gray. (B) Laser scanning microscope image of Lucifer yellow fluorescence loaded into an ORC isolated from a newt. In the picture, the ciliary image is thicker than the original diameter, because of the light deflection. The colored circle on a cilium indicates the area illuminated by a laser beam to photolyse caged cAMP previously loaded into the cell. Scale bar is 10 µm. (Modified from Takeuchi H and Kurahashi T, *J. Neurosci.*, 28, 766–75, 2008. With permission.)

TABLE 8.1
Intracellular and Extracellular Ion Concentrations at the Apical Part of ORCs
and Calculated Nernst Potentials

lon	[lon] <sub>in</sub> (mM)	[lon] <sub>out</sub> (mM)	E <sub>Nernst</sub> (mV)
Na <sup>+</sup>	$53 \pm 31$	$55 \pm 12$	+1
$K^+$	$172 \pm 23$	$69 \pm 10$	-24
Free Ca <sup>2+</sup>	$40 \pm 9 \text{ nM}$	4.8	+156
Cl-	$54 \pm 4$	$55 \pm 11$	0

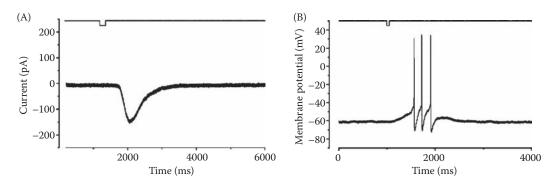
*Note:* Intracellular Ca<sup>2+</sup> measured in salamander cilia with a Ca<sup>2+</sup>-sensitive fluorescent dye (Leinders-Zufall T et al. 1997); extracellular Ca<sup>2+</sup> is the midpoint of the range (2.6–7.1 mM) determined with a Ca<sup>2+</sup>-sensitive microelectrode in olfactory mucus of rat (Crumling MA and Gold GH 1998). Intracellular Cl<sup>-</sup> measured in the knobs of rat ORCs with a Cl<sup>-</sup>-sensitive fluorescent dye (Kaneko H et al. 2004). The other Ionic concentrations are the total ionic concentrations measured by energy-dispersive x-ray microanalysis in the knobs of rat ORCs (Reuter D et al. 1998).

environment, the mucosal ionic condition (see Table 8.1) could be affected by the external conditions, such as the humidity. Possible changes in the ionic environment will be especially relevant for water-living animals. In contrast, the ionic composition of the interstitial solution is expected to be stable.

At the apical part of the ORCs, the dendritic tip is slightly swelled into a "terminal swelling" or "olfactory knob," from which cilia extend to expose their membrane to the external environment. The ORCs of lower vertebrates have about six motile cilia that can be as long as 200 µm (Menco 1980). The diameter of a frog cilium is about 0.28 µm near the base of the cilium and becomes 0.19 µm in the distal portion (Menco 1980). The ORCs of mammals have about 17 nonmotile cilia, ranging from 15 to 50 µm in length, with a diameter of about 0.11 µm in their distal portion (Lidow and Menco 1984; Menco 1997, 1980; Menco and Morrison 2003). The ciliary structure allows two important features: on one side, the presence of numerous fine cilia protruding from the olfactory knob greatly increases the surface area of the ORC that can be exposed to the external environment (Menco 1980) and produces an increase in the probability of interaction with odorant molecules, and on the other side, the long and thin structure of each cilium increases the ratio between the membrane surface area and the cytoplasmic volume. This feature is of great relevance in the transduction process, because in a small volume a limited variation in the number of molecules could produce a large concentration change. It should also be taken into account that the volume of the cilia available for the cytosol is further restricted by the ciliary cytoskeleton. Indeed, the cilia contain the 9+2 microtubules structure and toward the tip, where the diameter of cilia becomes thinner, its structure changes by lacking the surrounding nine microtubules (Kerjaschki 1976).

# 8.3 FUNCTIONS OF THE OLFACTORY CILIA

Several important physiological functions occur in the olfactory cilia, including odorant detection, generation of electrical excitation, signal amplification, adaptation or desensitization, and masking. An outline of each of these functions will be described in this section, while the molecular mechanisms underlying each of them will be discussed in the following sections.

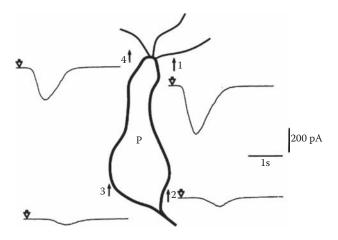


**FIGURE 8.2** Voltage and current responses induced by odorant stimulation. (A) Current responses evoked by odorant stimulation under the voltage-clamp condition with holding potential at -60 mV. After a short latency, the odorant stimulation induced a transient inward current. (B) Same cell as in (A) stimulated under the current-clamp condition. Odorant stimuli induced a slow depolarization and, in turn, initiated repetitive action potentials. Downward deflection of the top trace indicates the timing and duration of the odorant stimulation. The stimulus duration was 200 ms. (Modified from Tomaru A. and Kurahashi T. *J. Neurophysiol.*, 93, 1880–88, 2005. With permission.)

#### 8.3.1 ELECTRICAL EXCITATION

The origin of odorant perception is the chemical interaction of odorant molecules with ORCs that convert the chemical information into electrical signals carrying information about the external world to the brain (for detailed reviews, see Schild and Restrepo 1998; Kleene 2008). In isolated ORCs, the response to odorant stimuli in solution has been well characterized. The response has mostly been measured under voltage-clamp upon presentation of a brief pulse of odorant. As illustrated in Figure 8.2A, the odorant stimulation generates a transient inward receptor current that is expected to depolarize the neuron in situ. The response typically lasts 1 s or more. In amphibians, the latency between the arrival of the stimulus and the onset of the current ranges from 150 to 600 ms (Firestein et al. 1990, 1993; Firestein and Werblin 1987; Kurahashi 1989; Tomaru and Kurahashi 2005; Takeuchi and Kurahashi 2003). In the mouse and rat, the latency is at most 160 ms (Reisert and Matthews 2001; Grosmaitre et al. 2006). This shorter latency is observed even in the intact epithelium, which requires that odorants diffuse through the mucus (Grosmaitre et al. 2006). For a strong stimulus, the amplitude of the peak receptor current can reach 1 nA (Ma et al. 2003; Firestein et al. 1993; Lowe and Gold 1993). The resting membrane potential of the ORC is between -80 and -60 mV (see discussion, Lagostena and Menini 2003) and the transduction current induces a slow and graded receptor potential. When the receptor potential reaches the threshold level, it induces action potentials. Figure 8.2B shows action potentials in response to an odorant stimulus in an ORC in the current-clamp configuration. The amplitude of the receptor potential becomes larger as the odorant concentration increases, producing a higher frequency of action potentials that send information about the odorant concentration to the olfactory bulb (Ma et al. 1999; Imanaka and Takeuchi 2001).

Olfactory cilia were first indicated as the site of odorant detection on the basis of their location at the interface between the internal and the external side of the body and of their morphology (Usukura and Yamada 1978; Menco 1980). Subsequent physiological studies have clearly shown that the cilia play a central role for odorant detection (Kurahashi 1989; Takeuchi and Kurahashi 2008; Firestein et al. 1990; Lowe and Gold 1991). As illustrated in Figure 8.3, an odorant stimulus directed at the cilia of an isolated ORC produced large responses, whereas stimuli directed at the soma gave very small responses (Kurahashi 1989). Moreover, it has been shown that the molecular elements needed for olfactory transduction are concentrated at the ciliary membrane, as described in detail in the following sections. Therefore, the initial events of transduction take place in the cilia.



**FIGURE 8.3** Spatial distribution of sensitivity to an odorant stimulus. An isolated ORC was stimulated under voltage-clamp condition by a brief odorant pulse (50 ms duration indicated by open arrows) from a glass pipette (tip diameter, ~1  $\mu$ m) placed at numbered arrowheads (1–4). Holding potential: -43.7 mV. The amplitudes of current responses show that the odorant sensitivity is greatly restricted to the olfactory cilia. (Modified from Kurahashi T. *J. Physiol.*, 419, 177–92, 1989. With permission.)

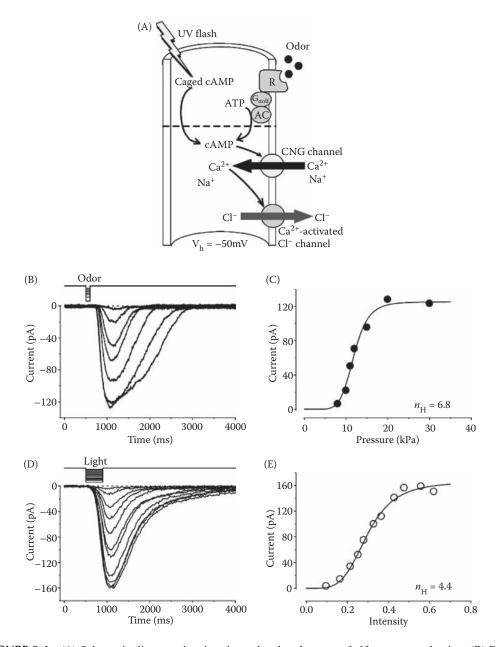
#### 8.3.2 SIGNAL AMPLIFICATION

Since the signal coming from the outside could be very small, receptor cells must have amplification mechanisms. In the olfactory cilia, two different types of signal amplification are present in the transduction cascade: (a) molar amplification and (b) nonlinear amplification. The former increases the number of active molecules, while the latter changes the input-output relation in a nonlinear way with a high cooperativity. The relation between odorant dose and peak receptor current is generally well fit by a Hill equation:  $I/I_{max} = C_{H}^{n}/(C_{H}^{n} + K_{1/2}^{n}H)$ , where  $I_{max}$  is the maximal current, *C* is the concentration of odorant,  $K_{1/2}$  is the half-maximal effective concentration, and  $n_{H}$  is the Hill coefficient, which corresponds to the cooperativity of the system. As shown in Figure 8.4B, in ORCs the Hill coefficient can reach values higher than 6. With such a nonlinear amplification, only a slight change in the concentration of odorant molecules produces a large change in the response.

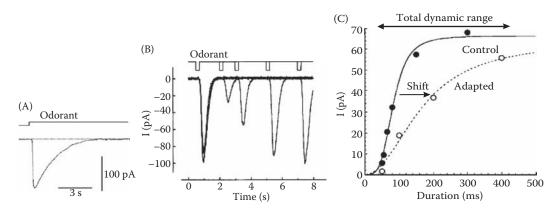
#### 8.3.3 Adaptation or Desensitization

Olfactory sensation gradually decreases during long or repeated exposures to odorant stimuli (see e.g., Getchell and Shepherd 1978). This phenomenon involves many processes along the entire olfactory pathway, but it begins in the cilia of ORCs. Indeed, during application of a prolonged odorant stimulus, the receptor current decreases with time, despite the continued presence of the stimulus, as shown in Figure 8.5A (Kurahashi and Shibuya 1990; Firestein et al. 1990; Menini 1995; Reisert and Matthews 1999; Zufall et al. 1991). On the other hand, when two brief odorant pulses are delivered within a short interval, the amplitude of the response to the second pulse is reduced (Kurahashi and Shibuya 1990; Kurahashi and Menini 1997). At the time of the second odorant stimulus, the cell is in an adapted state, the first (conditioning) pulse having desensitized the neuron to subsequent stimuli. The desensitization is greater with shorter interstimulus intervals as shown in Figure 8.5B (Kurahashi and Shibuya 1990; Kurahashi and Menini 1997; Takeuchi et al. 2003), and the current amplitude gradually recovers to the initial value, increasing the interval between odorant pulses.

Sensory adaptation is not merely a reduction in response amplitude, but its physiological role involves the adjustment of the response to allow a cell to work over a broad range of stimuli (for review, see Torre et al. 1995). Indeed, in odorant adaptation to repetitive stimuli there is a shift of the dynamic range (i.e., the range of stimulus concentrations over which the OSN is able to respond)



**FIGURE 8.4** (A) Schematic diagram showing the molecular elements of olfactory transduction. (B) Dose response to odorant stimulation in isolated newt ORCs under voltage-clamp condition (holding potential -50 mV). (C) Peak amplitudes of responses obtained in (B) were plotted against the dose of odorant. The smooth line was drawn by fitting of the data points by the Hill equation, with a Hill coefficient ( $n_{\rm H}$ ) of 6.8. (D) An isolated newt ORCs under voltage-clamp condition was loaded with 1 mM of caged cAMP and stimulated with various ultraviolet light intensities. Downward deflections of the top trace in (B) and (C) indicate the timing and duration of the stimulation. (E) Peak amplitudes of responses obtained in (D) were plotted against light intensity. The smooth line was drawn by fitting of the data points by the Hill equation, with a Hill coefficient ( $n_{\rm H}$ ) of 4.4. (Modified from Takeuchi H. and Kurahashi T. *J Neurosci.*, 25, 11084–91, 2005. With permission.)



**FIGURE 8.5** Adaptation in olfactory receptor cells. (A) Adaptation during prolonged odorant stimulation. An isolated ORC was stimulated under voltage-clamp condition by a long odorant pulse. The transduction current declines despite the continuous presence of the odorant molecules. (B) Adaptation during repeated odorant stimulation. In each recording, two brief odorant pulses were delivered to ORCs under voltage clamp with different interstimulus intervals. With shorter intervals, the response to the second stimulation shows a greater reduction. Downward deflections of the top trace in (B) indicate the timing and duration of the stimulation. (C) Dose-response to odorant stimulation in control and adapted state. In the adapted state, the ORC shows a shift in odorant sensitivity to a higher dose and a decrease of the slope of the dose-response curve, allowing an increase of the total dynamic range of the response. ((A) Modified from Kurahashi T and Shibuya T. *Brain Res.*, 515, 261–68, 1990; (B), (C) Modified from Kurahashi T. and Menini A. *Nature.*, 385, 725–29, 1997.)

toward higher odorant concentrations compared with the control state (Kurahashi and Menini 1997; Boccaccio et al. 2006; Reisert and Matthews 1999). In the adapted state, a stronger stimulus is required to produce a half-maximal response with respect to control condition (Figure 8.5C). During a prolonged exposure to an odorant, adaptation is expected to continuously reset the neuron to discriminate higher odorant concentrations without saturating the transduction process.

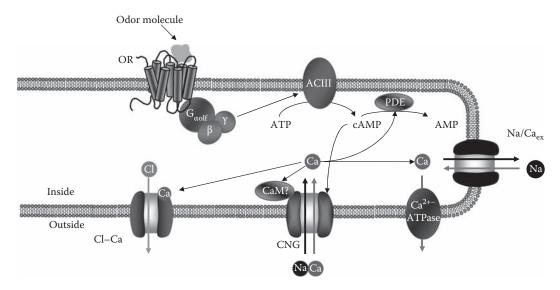
# 8.3.4 MASKING

In human history, odorants have been widely used to induce pleasant smells. At the same time, some odorants (like flavors and fragrances) have also been used for masking unpleasant smells. In particular, a wide-spectrum odorant suppression has been employed in the smell-masking industries, such as the usages of spices and the development of perfumes. It has been suggested that the masking effect begins at the receptor cell level. The response from the single ORC is suppressed by the stimulant itself or by additional odorants (Kurahashi et al. 1994). The significance and the mechanisms of the olfactory suppression will be explained in Section 8.5.4.

# 8.4 MOLECULAR MECHANISMS OF OLFACTORY TRANSDUCTION

#### 8.4.1 Odorant Receptors and Cyclic Adenosine Monophosphate (cAMP) Formation

A large majority of ORCs express a member of the odorant receptor family discovered by Buck and Axel (1991). Odorant receptors belong to the superfamily of G-protein-coupled receptors and have the same general structure with seven hydrophobic membrane-spanning regions, but they differ in their amino acid sequence, especially in transmembrane domain III, IV, and V, suggesting that these parts are responsible for the discrimination of odorant species (Mombaerts 2004). In the mouse, there are about 1000 genes encoding different types of odorant receptors. Each ORC expresses only one type of odorant receptor gene in its ciliary membrane. The reader is referred to Chapter 7 of this book for a detailed discussion about odorant receptors.



**FIGURE 8.6** Molecular mechanisms of olfactory transduction. The binding of odorant molecules to an odorant receptor (OR) induces the G-protein-mediated activation of adenylyl cyclase. cAMP directly gates CNG channels, generating a depolarizing influx of Na<sup>+</sup> and Ca<sup>2+</sup>. The increase in intraciliary Ca<sup>2+</sup> mediates both excitatory and inhibitory events. Ca<sup>2+</sup> gates a Cl<sup>-</sup> channel that produces a depolarizing efflux of Cl<sup>-</sup> (Cl-Ca). On the other hand, Ca<sup>2+</sup>, through calmodulin (CaM) and/or other Ca<sup>2+</sup>-binding proteins, mediates the reduction of cAMP sensitivity of CNG channels and activates the PDE that hydrolyzes cAMP to AMP. Intraciliary Ca<sup>2+</sup> concentration returns to basal level through the activity of a Na<sup>+</sup>/Ca<sup>2+</sup>-exchanger and Ca<sup>2+</sup>-ATPase.

A single ORC responds to many types odorants and, on the other hand, a single type of odorant can stimulate several types of ORCs expressing different odorant receptors (Firestein et al. 1993). The random distribution of odorant receptors and their broad selectivity are characteristics of olfactory coding at the level of the olfactory epithelium.

As shown in Figure 8.6, the ligand-bound receptor activates an olfactory-specific excitatory G-protein,  $G_{colf}$  (Jones and Reed 1989), whose structure is similar to that of other types of G-proteins. It consists of three subunits,  $\alpha$ ,  $\beta$ , and  $\gamma$ , at the cytoplasmic surface of the ciliary membrane. By homology with other G-proteins, it is likely that after the odorant binds to a receptor,  $G_{\alpha olf}$  exchanges guanosine 5'-diphosphate (GDP) for guanosine 5'-triphosphate (GTP), and the GTP-bound  $G_{\alpha olf}$  subunit dissociates from the  $\beta$  and  $\gamma$  subunits and activates AC type III (Bakalyar and Reed 1990). This is an integral membrane protein with 12 transmembrane domains, both C- and N-terminals at the intracellular side, and the catalytic domain located between transmembrane domain 6 and 7. The catalytic region of AC converts adenosine triphosphate (ATP) into cAMP, a molecule that plays a fundamental role in olfaction as a second messenger and mediates signal transduction for a wide variety of odorants (Brunet et al. 1996; Lowe et al. 1989; Takeuchi and Kurahashi 2003). cAMP is a small and water-soluble molecule, with a molecular weight of 329. The diffusion coefficient of cAMP in frog olfactory cilia was estimated to be  $2.7 \times 10^{-6}$  cm<sup>2</sup> s<sup>-1</sup> (Chen et al. 1999), which provides an average onedimensional diffusion speed of approximately 20 µm/s. If cAMP is generated at the tip of the olfactory cilia, it could diffuse through the ciliary cytoplasm to the base of the cilia during the odorant response, which typically lasts a couple of seconds (Figure 8.4B). In the olfactory cilia, the actual spread of cAMP seems to be limited (Takeuchi and Kurahashi 2008). This is probably due to several factors, including the binding of cAMP to CNG channels and the activity of the phosphodiesterase PDE1C2, expressed almost exclusively in the cilia (Borisy et al. 1992; Yan et al. 1995).

#### 8.4.2 CYCLIC NUCLEOTIDE-GATED CHANNELS

CNG channels are the mediators of the chemoelectrical energy conversion in olfactory cilia. Indeed, information about odorant molecules is first transmitted as chemical information and then, at the level of the CNG channel, is converted into an electrical signal by the activation of ion fluxes across the plasma membrane. This membrane protein consists of four subunits containing two CNGA2, one CNGA4, and one CNGB1b (Zheng and Zagotta 2004). Each subunit has six transmembrane domains (S1–S6), and a pore region between S5 and S6 domains. A cyclic nucleotide-binding site is located near the C-terminal at the cytoplasmic side in each subunit (Dhallan et al. 1990), for a total of four binding sites per each CNG channel. The cAMP binding and channel activation show an allosteric effect, by which the CNG channel displays cooperativity (Hill coefficient of 2). Recently, along this line, Nache et al. (2005) and Biskup et al. (2007) demonstrated that in homomeric CNGA2 channels the binding of the second cAMP molecule brings the channel almost to its maximum open probability of about 0.7–0.8 (Kurahashi and Kaneko 1993; Kleene 1997; Larsson et al. 1997; Reisert et al. 2003).

It is significant to note that CNG channels are permeable to monovalent ions, such as Na<sup>+</sup> and K<sup>+</sup>, and also to Ca<sup>2+</sup> ( $P_{Ca}/P_{Na} = 6.5$ ; Kurahashi and Shibuya 1990). The fraction of current carried by Ca<sup>2+</sup> was estimated to be 0.4 in heterologously expressed rat CNG channels in the presence of 2 mM of extracellular Ca<sup>2+</sup> at -70 mV (Dzeja et al. 1999). This Ca<sup>2+</sup> influx results in an increase of Ca<sup>2+</sup> in the intraciliary medium and this has important roles in olfactory transduction.

The unitary conductance of the single CNG channel is about 30 pS in the absence of divalent cations, but this value becomes smaller when the external solution contains  $Ca^{2+}$  or  $Mg^{2+}$  ions (Zufall and Firestein 1993). Indeed,  $Ca^{2+}$  and  $Mg^{2+}$  entry at negative potentials produces an open channel block causing an increase in flickering activity of the channel. This temporary block reduces the current carried by all cations, producing a very small, single channel conductance (~ 1.5 pS, Zufall and Firestein 1993; 0.56 pS, Kleene 1997). A small, single channel conductance plays a relevant physiological role, since, by using a large number of tiny events, the integrated current has a high signal-to-noise ratio (Kurahashi and Kaneko 1991; for review see Kleene 2008).

The channel density in the cilium has been estimated by electrophysiological methods with widely differing results: 1750 channels/ $\mu$ m<sup>2</sup> in the toad (Kurahashi and Kaneko 1993), 67–202 channels/ $\mu$ m<sup>2</sup> in the frog (Kleene 1994; Larsson et al. 1997), and 8 channels/ $\mu$ m<sup>2</sup> at the dendritic knob/ cilia in the rat (Reisert et al. 2003).

#### 8.4.3 CALCIUM-ACTIVATED CHLORIDE CHANNELS

 $Ca^{2+}$  ions entering the cilia through CNG channels play crucial roles in olfactory transduction; namely the activation of  $Cl_{(Ca)}$  channels and the regulation of adaptation (for review, see Matthews and Reisert 2003; Menini 1999). Lateral (longitudinal) diffusion of  $Ca^{2+}$  seems to be restricted, because the responses to double pulse local laser stimuli applied to different parts along the cilium are independent, and do not show  $Ca^{2+}$ -dependent adaptation (Takeuchi and Kurahashi 2008; for adaptation see Section 8.5.2). Within the cilia,  $Ca^{2+}$  ions are buffered by  $Cl_{(Ca)}$  channels, calmodulin (CaM), and some unidentified  $Ca^{2+}$ -binding proteins (see, e.g., Uebi et al. 2007).

Olfactory signal transduction has the peculiarity that electrical excitation is generated by the activation of two different types of ion channels: CNG and  $Cl_{(Ca)}$  channels (Figure 8.6).  $Cl_{(Ca)}$  channels are present in the ciliary membrane and are activated by a rise in ciliary Ca<sup>2+</sup> concentration (Kleene 1993; Kleene and Gesteland 1991; Lowe and Gold 1993; Kurahashi and Yau 1993). Moreover, as shown in Table 8.1 and discussed in Section 8.4.4, ORCs maintain an elevated intracellular Cl<sup>-</sup> concentration that is in the same range as the Cl<sup>-</sup> concentration present in the mucus at the external side of the cilia (Kaneko et al. 2001; Reuter et al. 1998). Therefore, in physiological conditions, the opening of  $Cl_{(Ca)}$  channels in the ciliary membrane causes an efflux of Cl<sup>-</sup> ions from the cilia, corresponding to an inward current that contributes further to the depolarization of these neurons.

It has been suggested that the presence of a pair of cationic and anionic currents in the depolarizing response to odorants is useful because the Cl<sup>-</sup> current produces a large amplification of the primary cationic CNG current (Lowe and Gold 1993) and the amplified signal has a higher signal-to-noise ratio than the primary signal (Kleene 1997). Therefore, in olfactory transduction, the secondary  $Cl_{(Ca)}$  channels ensure a high-gain and low-noise amplification of the primary CNG current, contributing up to 90% of the total odorant-induced current (Boccaccio and Menini 2007; for reviews, see Kleene 2008; Frings et al. 2000, 2009).

The functional properties of the  $Cl_{(Ca)}$  conductance have been investigated under various conditions with a variety of electrophysiological techniques, each revealing important information about the channel properties. The electrical activity in response to odorants, which can be recorded at the surface of the olfactory epithelium as a negative electrical field potential, the electro-olfactogram (EOG) (Ottoson 1955; Scott and Scott-Johnson 2002) has been shown to be primarily caused by the depolarizing action of Cl<sup>-</sup> current, since more than 80% of the response can be blocked by niflumic acid (NFA), a blocker for Cl<sup>-</sup> channels (Nickell et al. 2006). The large contribution of the Cl<sup>-</sup> conductance to the transduction current is confirmed by experiments in isolated cells obtained with the suction pipette or in the whole-cell voltage clamp configuration, showing that the fraction of the current carried by Cl<sup>-</sup> can be up to 90% in mice (Boccaccio and Menini 2007; Reisert et al. 2005). The activation of the conductance by Ca<sup>2+</sup> has been carefully investigated in inside-out excised membrane patches from the knob/cilia of mouse ORCs. Studies in the mouse (Reisert et al. 2005; Pifferi et al. 2006b) have shown that the dose-response relation is well fit by the Hill equation with half maximal activation between 2.2 and 4.7  $\mu$ M Ca<sup>2+</sup>, and Hill coefficient between 2.0 and 2.8. Moreover, this conductance shows a Ca<sup>2+</sup>-dependent inactivation, which is reversible after a few seconds removal of  $Ca^{2+}$  (Reisert et al. 2003, 2005), but also an irreversible run-down indicating that some modulatory component of the channel may be lost after the excision of the membrane (Reisert et al. 2003, 2005). The native olfactory  $Cl_{(Ca)}$  channel is apparently not affected by  $Ca^{2+}$ -CaM (Reisert et al. 2003; Kleene, 1999) and, at present, no modulators of the channel activity are known.

The current conducted by a single olfactory Cl<sup>-</sup> channel is so small that single-channel studies have not been possible. By noise analysis of macroscopic currents, the unit conductance was estimated to be 1.3 pS in the rat (Reisert et al. 2003) and 1.6 pS in the mouse (Pifferi et al. 2006b), with a channel's maximum open probability of .97. The combination of a very small single channel conductance, together with a high maximum open probability, allows a high amplification of the primary current without an increase of noise (Kleene 1997).

Specific blockers for  $Cl_{(Ca)}$  with high binding affinity are not available, but some commonly used extracellular blockers of  $Cl_{(Ca)}$  include NFA and 4-acetamido-4'-isothiocyanato-stilben-2,2'-disulfonate (SITS) (Lowe and Gold 1993; Kleene 1993; Kurahashi and Yau 1993; for review, see Frings et al. 2000).

The molecular identity of  $Cl_{(Ca)}$  channels is still elusive, although some proteins have been proposed as possible candidates for being a molecular component of the conductance, including bestrophin-2 (mBest2) and TMEM16B (ANO2) (Pifferi et al. 2006b; Pifferi et al. 2009; Stephan et al. 2009). Pifferi et al. (2006b) showed that mBest2 mRNA is expressed in ORCs, and that the protein is expressed in the cilia of ORCs, where it colocalizes with CNGA2, the principal subunit of the olfactory CNG channel responsible for the primary transduction current (Pifferi et al. 2006b). Moreover, the functional properties of the current induced by heterologous expression of mBest2 and those of the native  $Cl_{(Ca)}$  channels from the dendritic knob/cilia of mouse ORCs have many similarities, but also some differences (Pifferi et al. 2006b). Indeed, the two currents have the same anion permeability sequence, small estimated single-channel conductances, and the same side-specific blockage by two Cl<sup>-</sup> channel blockers (NFA and SITS). The most significant difference between the two currents is the sensitivity to intracellular Ca<sup>2+</sup>. In fact, currents are halfmaximal at a Ca<sup>2+</sup> concentration of 0.4  $\mu$ M for mBest2, whereas native currents require a higher Ca<sup>2+</sup> concentration, 4.7  $\mu$ M. Very recently, it has been proposed that the anoctamin/TMEM16 family of membrane proteins are Cl<sub>(Ca)</sub> channels (Caputo et al. 2008; Yang et al. 2008; Schroeder et al. 2008). Interestingly, a previous study showed that *Tmem16b* is expressed in the mature sensory neurons of the olfactory epithelium by *in situ* hybridization (Yu et al. 2005). Proteomic screenings of ciliary membranes recently revealed that TMEM16B is a prominent protein in the olfactory cilia (Mayer et al. 2009; Stephan et al. 2009). Moreover, Stephan et al. (2009) showed that the fusion protein TMEM16B-EGFP localized to the cilia when expressed *in vivo* using an adenoviral vector. A comparison of the properties of TMEM16B-induced currents upon heterologous expression in a mammalian cell line with those of native  $Cl_{(Ca)}$  in the sensory neurons of the olfactory epithelium indicates that the two channels are remarkably similar (Pifferi et al. 2009; Stephen et al. 2009). At present, antibodies against Tmem16b are not available, therefore, it is not possible to establish whether the protein is expressed in the cilia. Future studies combining a multidisciplinary approach from genetic, molecular biology and electrophysiology will be necessary to reveal the involvement of mBest2, TMEM16B, and other candidates in the olfactory  $Cl_{(Ca)}$ .

#### 8.4.4 ION HOMEOSTASIS

Since electrical excitation is due to ion fluxes across the cell membrane, the ion homeostasis is very important in olfactory signal transduction. The concentration of Ca2+ inside the cilia increases during odorant stimulation and is restored to basal levels by two major mechanisms: extrusion of Ca<sup>2+</sup> by Na<sup>+</sup>/Ca<sup>2+</sup> exchanger and Ca<sup>2+</sup>-ATPase (Figure 8.6). The indication of the presence of a Na<sup>+</sup>/Ca<sup>2+</sup> exchanger in the dendrite, dendritic knob, and possibly in the cilia, was initially proposed by Jung et al. (1994) and Noe et al. (1997), and was confirmed physiologically by Reisert and Matthews (1998) on the basis of ion substitution experiments. Indeed, it is well known that the Na<sup>+</sup> driving force establishes  $Ca^{2+}$  extrusion by the Na<sup>+</sup>/ $Ca^{2+}$  exchanger and that the substitution of Na<sup>+</sup> bathing the cilia with another cation, such as choline<sup>+</sup> or Li<sup>+</sup>, known for inhibiting the exchanger, should inhibit Ca<sup>2+</sup> efflux. Reisert and Matthews (1998) measured the time course of the Cl<sub>(Ca)</sub> current component with the suction (loose seal) electrode. Since the  $Cl_{(Ca)}$  current is activated by the presence of cytoplasmic Ca2+, the time course of Cl<sub>(Ca)</sub> represents an index of the intracellular Ca2+ concentration. They observed that the current response was greatly prolonged in the absence of external Na<sup>+</sup>, which indicates that Ca<sup>2+</sup> was not extruded, suggesting that the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger is the main mechanism that returns cytoplasmic Ca<sup>2+</sup> concentration to basal levels after stimulation. Furthermore, Reisert et al. (2003) showed that the Na<sup>+</sup>-dependent Ca<sup>2+</sup> extrusion can also be detected in the excised patch membrane obtained from the apical dendrite. Antolin and Matthews (2007) further investigated the Na<sup>+</sup>-dependence of the exchanger in the frog and found that the rate of Ca<sup>2+</sup> extrusion was only modestly affected by extracellular Na<sup>+</sup> until its concentration was 30% of its value in Ringer solution, suggesting that Ca<sup>2+</sup> extrusion from the cilia would be expected to be only marginally affected by modest changes in mucus Na<sup>+</sup> concentration.

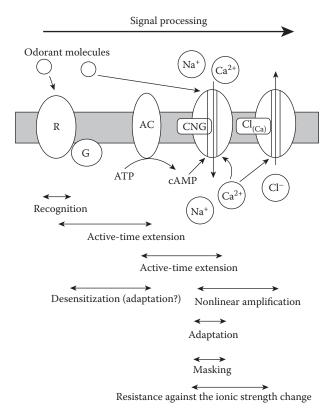
The olfactory Na<sup>+</sup>/Ca<sup>2+</sup> exchanger seems to be independent of potassium ion (Reisert et al. 2003), different from the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger of rod photoreceptor cells, in which the potassium ion is cotransported. However, Pyrski et al. (2007) reported that ORCs also express the gene encoding for the potassium-dependent Na<sup>+</sup>/Ca<sup>2+</sup> exchangers (NKCX1-3), although at present there is no functional evidence that they are involved in Ca<sup>2+</sup> clearance during olfactory transduction.

Another major mechanism that reduces the intraciliary  $Ca^{2+}$  concentration is the activity of  $Ca^{2+}$ -ATPase. Several isoforms of  $Ca^{2+}$ -ATPase have been localized to the cilia by immunohistochemistry (Castillo et al. 2007; Weeraratne et al. 2006). Moreover, Castillo et al. (2007) showed that, in rat and toad ORCs, the current relaxation time-course of the cAMP-induced response (induced by the photolysis of cytoplasmic caged cAMP) is prolonged in the absence of intracellular ATP and by blocking the  $Ca^{2+}$ -ATPase by carboxyeosin. These results may indicate that  $Ca^{2+}$ -ATPase is also involved in  $Ca^{2+}$  extrusion from the cilia.

In the nervous system, though openings of Cl<sup>-</sup> channels usually produce inhibitory responses in neurons, as exemplified by the GABAergic and glycinergic synapses, in ORC the Cl<sup>-</sup> current is excitatory and amplifies the primary transduction current. To generate an excitatory Cl<sup>-</sup> current, ORCs have Cl<sup>-</sup> uptake systems to maintain a high intracellular Cl<sup>-</sup> concentration. Using 2-photon ion imaging, Kaneko et al. (2004) reported that the uptake mechanism of Cl<sup>-</sup> resides in the apical membrane of ORCs, most probably within the ciliary membrane or in the membrane of the dendritic knobs. Moreover, Reisert et al. (2005) showed that a Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>-</sup> cotransporter (NKCCl) greatly contributes to Cl<sup>-</sup> homeostasis in the ORC, since ORCs from mice lacking NKCC1 are not able to maintain the high intracellular Cl<sup>-</sup> concentration necessary to sustain the excitatory Cl<sub>(Ca)</sub> current. By immunohistochemistry, NKCC1 was localized in the soma and in the dendrite, but not in the cilia (Reisert et al. 2005). It is likely that additional Cl<sup>-</sup> accumulation systems are present. Indeed, Nickell et al. (2007) showed that multiple chloride transporter systems are expressed in the mouse olfactory epithelium. At present, the mechanisms for the Cl<sup>-</sup> homeostasis in the ORC and its cilia are still unclear.

# 8.5 INTERACTIONS BETWEEN CILIARY ION CHANNELS AND CYTOSOLIC MESSENGERS

Olfactory transduction occurs in the olfactory cilia by means of the molecular elements described above. These molecules with their own integrative properties shape the outline of the sensory characteristics, and their different functional roles are illustrated in Figure 8.7. Odorant molecules bind to odorant receptors and the binding switches on the transduction cascade. It has been indicated that the odorant dwell-time on the receptor is at most of the order of 1 ms (Bhandawat et al. 2005), but the active signaling time is extended at the level of the G-protein and AC to several hundreds of milliseconds at upstream points to AC (Takeuchi and Kurahashi 2002). Furthermore, even after



**FIGURE 8.7** Functional roles of molecular elements of olfactory transduction. Schematic diagram showing the involvement of the different molecular elements in the shaping of the characteristic features of olfactory transduction.

the inactivation of AC, the signal remains as cytoplasmic cAMP, cAMP-CNG complex, intraciliary  $Ca^{2+}$ , and  $Ca-Cl_{(Ca)}$  complex. Such a time extension produces a molar amplification. In fact, the opening of only a 0.01 pA single CNG channel for 1 ms allows the entry of 70 Na<sup>+</sup> ions in the cilia. At the CNG and  $Cl_{(Ca)}$  channels, the cooperativity is increased to 6, as illustrated in Figure 8.4B, showing a nonlinear amplification. Because of this, a single ORC can detect a very small change in odorant concentration and the response becomes almost all-or-nothing.

The combination of CNG and  $Cl_{(Ca)}$  channels carrying ions in opposite directions has the important physiological advantage of making the olfactory response resistant against possible changes in the ionic composition surrounding the cilia (Kleene and Pun 1996).

Short-term adaptation is regulated by the  $Ca^{2+}$  feedback to the CNG channel, and also at some point, not yet identified, before activation of AC (Takeuchi and Kurahashi 2002). Finally, olfactory masking is regulated at the CNG channel level by the direct odorant suppression of the channel (Chen et al. 2006), whereas the  $Cl_{(Ca)}$  channel is not involved in this process (Takeuchi et al. 2009).

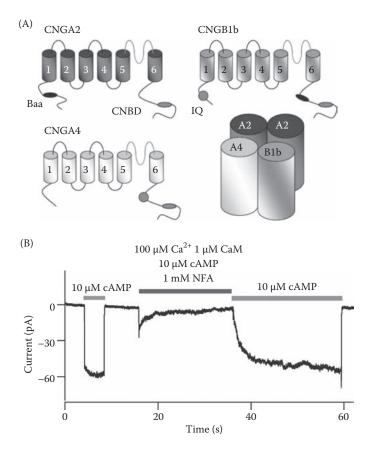
#### 8.5.1 MOLECULAR MECHANISMS OF ODORANT ADAPTATION IN THE CILIA

As described in Section 8.3.3, the ORC itself has the ability to adapt. This sensory adaptation occurs in the ciliary membrane and it depends on Ca<sup>2+</sup> entry through CNG channels (Kurahashi and Shibuya 1990). Kurahashi and Menini (1997) investigated the localization of the principal molecular mechanism for adaptation in the olfactory transduction process. To determine whether the response reduction in the adapted state was attributable to a reduction in the cAMP production or was instead due to other processes occurring after the production of cAMP, CNG channels in intact neurons were directly activated by flash photolysis of caged cAMP. The ciliary cytoplasm was loaded with caged cAMP through diffusion from a patch pipette, and application of ultraviolet flashes to the cilia caused the photorelease of various cAMP concentrations. Therefore, cAMP-gated channels can be directly activated, bypassing the early stages of odorant transduction (i.e., receptor activation and G-protein and adenylate cyclase signaling). cAMP and odorant-induced responses were found to have similar adaptation properties, indicating that the entire adaptation process takes place after the production of cAMP (Kurahashi and Menini 1997; Menini 1999; but see also Takeuchi and Kurahashi 2002).

By using a hydrolysis-resistant caged cAMP analog, caged 8Br-cAMP, Boccaccio et al. (2006) have shown that the hydrolysis of cAMP by PDE is not involved in adaptation. Furthermore,  $Cl_{(Ca)}$  channels have been shown to be unrelated to olfactory adaptation (Boccaccio et al. 2006; Kurahashi and Menini 1997).

Several data show that CNG channels are modulated by a  $Ca^{2+}$ -dependent feedback. In native CNG channels, it has been demonstrated that the addition of micromolar concentrations of intracellular  $Ca^{2+}$  is able to decrease the channel sensitivity to cAMP, probably by activating a  $Ca^{2+}$ -responsive endogenous factor already preassociated with the channel (Kramer and Siegelbaum 1992; Lynch and Lindemann 1994; Balasubramanian et al. 1996; Bradley et al. 2004). Bradley et al. (2004) have shown that  $Ca^{2+}$ -free CaM, called apocalmodulin, is able to bind to the heterologously expressed heteromeric olfactory CNG channels even in the absence of  $Ca^{2+}$ . Moreover, when  $Ca^{2+}$  concentration rises above 100 nM,  $Ca^{2+}$  can rapidly modulate the CNG channel sensitivity by binding directly to the preassociated CaM. Furthermore, it was suggested that in native channels also, the preassociated endogenous factor could be apocalmodulin, although a direct demonstration is still missing (Bradley et al. 2004). Since  $Ca^{2+}$  enters into the olfactory cilia through the CNG channel itself, the preassociated  $Ca^{2+}$  responsive factor could provide a very fast feedback modulation at the channel level.

Early works (Chen and Yau 1994; Liu et al. 1994; Varnum and Zagotta 1997; Grunwald et al. 1999; Zheng et al. 2003) identified in the N-terminus of CNGA2 a classic basic amphiphilic  $\alpha$ -helix (Baa) motif with high affinity for Ca<sup>2+</sup>-CaM and showed that the sensitivity to cAMP of heterologously



**FIGURE 8.8** CNG modulation and adaptation in ORCs. (A) Topological model and assembly of subunits of the olfactory CNG channel. Each transmembrane domain is indicated by a number, the pore loop is located between 5 and 6. The cyclic nucleotide-binding site (CNBD) is located in the C-terminal domain. Calmodulinbinding sites of the calcium-dependent "Baa type" are represented in black, whereas the calcium-independent "IQ-type" are in gray. (B) Native olfactory CNG channels are inhibited by Ca<sup>2+</sup>-calmodulin (CaM) in excised inside-out patches. A patch was exposed to 10  $\mu$ M cAMP in a solution containing nominally 0 Ca<sup>2+</sup>. Then, the same patch was exposed to a solution containing, in addition to 10  $\mu$ M cAMP, 1  $\mu$ M calmodulin and 67  $\mu$ M Ca<sup>2+</sup> (in the presence of 1 mM NFA to block native Cl<sub>(Ca)</sub> current). The addition of Ca<sup>2+</sup>-CaM quickly inhibited the cAMP-gated current, which slowly recovered to its initial value after removal of Ca<sup>2+</sup>-CaM. (Modified from Pifferi S, Boccaccio A., and Menini A. *FEBS Lett.*, 580, 2853–59, 2006a. With permission.)

expressed homomeric CNGA2 channels was decreased by the binding of  $Ca^{2+}$ -CaM to the Baa motif (Figure 8.8A). However, in recent years, there has been considerable progress in elucidating the molecular events producing modulation of the native channels and it has been shown that the Baa motif of CNGA2 does not play any role in  $Ca^{2+}$ -CaM modulation of heteromeric channels. On the contrary, by comparing properties of native channels with heterologously expressed heteromeric channels, the modulatory subunits CNGA4 and CNGB1b have been shown to be responsible for the physiological modulation of  $Ca^{2+}$ -CaM (Bradley et al. 2001, 2004). Munger et al. (2001), in excised patches containing native heteromeric olfactory CNG channels, measured a fast current inhibition on the addition of  $Ca^{2+}$ -CaM, which persisted for several seconds also after CaM was removed in  $Ca^{2+}$ -free solution (Figure 8.8B). In contrast, homomeric CNGA2 showed a slower onset of inhibition by  $Ca^{2+}$ -CaM and a faster recovery, suggesting that CNGB1 and CNGA4 mediated the physiological relevant modulation of the channel (Bradley et al. 2001).

Indeed, the modulatory subunits also have CaM-binding sites: CNGA4 has an IQ-type CaMbinding site located at the C-terminal region, while CNGB1b has a similar IQ-type site located at the N-terminal region and a Baa motif in the C-terminal region (Figure 8.8A). It has been shown that the IQ-type sites are necessary and sufficient for  $Ca^{2+}$ -CaM channel inhibition, whereas the Baa-type site is not necessary (Bradley et al. 2001, 2004). Moreover, ORCs from knockout mice for CNGA4 showed a reduced adaptation both after prolonged odorant stimulation and with double pulse protocol (Munger et al. 2001). However, these data have been partially challenged by a subsequent report showing that the lack of CNGA4 or CNGB1 greatly reduced the sensitivity of the channel to cAMP and also impaired the trafficking of subunits to olfactory cilia (Michalakis et al. 2006). Indeed, Song et al. (2008) produced mice with CNG channels lacking the IQ-type CaMbinding site in the CNGB1 subunit, which should make the channels insensitive to CaM modulation, but with normal trafficking and cAMP sensitivity. They reported that adaptation to double stimulation was not affected by the binding site deletion, suggesting that CaM did not play a pivotal role in this process. However, in this mouse line, the response to odorants shows a longer termination both after brief and long-lasting odorant stimulations, pointing out the role of Ca<sup>2+</sup>-CaM modulation in shaping the termination of the odorant response (Song et al. 2008).

These results could also suggest that CaM is not the Ca<sup>2+</sup>-responsive factor that is coassembled with the CNG channel (Bradley et al. 2001). Other experimental evidence argue against this hypothesis, in particular the endogenous factor appears to bind to the CNG channels in a very stable manner, being washed away only after intense rinsing in Ca<sup>2+</sup>-free solution (Kramer and Siegelbaum 1992; Kleene 1999; Bradley et al. 2004). However, it is also possible to speculate that the binding of "native" CaM is more stable because the channel or the CaM itself undergoes post-transductional modifications that change the properties of the interaction. On the other hand, it cannot be excluded that other proteins, in addition to CaM, contribute to the Ca<sup>2+</sup>-mediated modulation of olfactory CNG channels (Pifferi et al. 2006a).

#### 8.5.2 SPATIAL ARRANGEMENTS OF TRANSDUCTION CHANNELS

To understand the signal transmission process between CNG and  $Cl_{(Ca)}$  channels, including nonlinear processes mediated by cytoplasmic Ca<sup>2+</sup>, it is important to know their spatial arrangements along the cilium. Localization of CNG channels along the cilium has been investigated both with electrophysiological and with electron microscopy immunogold, while for the distribution of  $Cl_{(Ca)}$ channels, only electrophysiological techniques have been used, because the molecular identity of these channels is still unknown.

#### 8.5.2.1 Distribution of Cyclic Nucleotide-Gated Channels

Nakamura and Gold (1987) performed the first experiments showing that CNG channels are present in the ciliary membrane, by directly activating the channels with cAMP in inside-out excised patches from the toad ciliary membrane. Kurahashi (1990) demonstrated that these channels are mainly localized in the cilia in whole-cell experiments, allowing cAMP to diffuse from the patch pipette into the cell interior. He observed that the response became larger and its latency shorter when the patch pipette was situated at the apical dendrite than when it was positioned at the soma. When examined with excised inside-out patches, the density of CNG channels in the newt was 1000 channels/ $\mu$ m<sup>2</sup> at the cilia, while in the dendrosomatic membrane there were only a few channels/ $\mu$ m<sup>2</sup> (Kurahashi and Kaneko 1991). This polarized distribution of CNG channels is consistent with the observation that odorant sensitivities are high at the cilia (Figure 8.3). The density of CNG channels has also been measured in the frog using a detached cilia preparation (Larsson et al. 1997). The current fluctuations accompanied with the activation of CNG channels were analyzed in non-space-clamped cilia and the channel density was estimated to be 70 channels/ $\mu$ m<sup>2</sup>.

The distribution of CNG channels along the cilia was investigated by electron microscopy immunogold against CNGA2 and with electrophysiological techniques. Electron microscopy showed that CNGA2 was predominantly localized at the tip of the cilia (Matsuzaki et al. 1999). Flannery et al. (2006) used the detached inside-out ciliary preparation and a cAMP diffusion model, and found that the proximal segment, which in the frog is the first 20% of the cilium, appears to express a small fraction of the CNG channels, whereas the distal segment contains their majority, mostly clustered in one region. Furthermore, Takeuchi and Kurahashi (2008) used submicron local laser spot to photolyze cytoplasmic caged cAMP (see Figure 8.1B), and examined the localization of the cAMP-induced current. Since a response was obtained everywhere along the single cilium, CNG channels were considered to be broadly distributed along the cilium. Local responses were therefore amplified by the high density of CNG channels, eliciting >100 pA current with a stimulus illuminating only 1  $\mu$ m length cilium (Takeuchi and Kurahashi 2008). This high current value is surprising if we consider that, given the very high input resistance of ORCs, 1 pA is sufficient to generate action potentials (Lynch and Barry 1989) that transmit the olfactory information to the brain.

#### 8.5.2.2 Distribution of Cl<sub>(Ca)</sub> Channels

Kleene and Gesteland (1991) first discovered the presence of a large  $Cl_{(Ca)}$  current in the detached cilia preparation. With noise analysis, Larsson et al. (1997) showed that the density of  $Cl_{(Ca)}$  channels in the cilia of frog ORCs is almost comparable to that of the CNG channels (70 channels/µm<sup>2</sup>). In contrast, Reisert et al. (2003) reported that in rat ORCs the density of  $Cl_{(Ca)}$  channels at the knob/ cilia was about eightfold bigger than that of the CNG channels (62 vs 8 channels/µm<sup>2</sup>).

Takeuchi et al. (2009) employed laser photolysis of intraciliary caged  $Ca^{2+}$ , and investigated the localization of  $Cl_{(Ca)}$  along the cilia. The channel distribution was broad along the cilia, displaying a spatial pattern similar to that of CNG channels. The broad distribution of  $Cl_{(Ca)}$  channels provides evidence that all molecular elements involving electrical excitation are distributed along the cilia. This is consistent with the theoretical estimation by Reisert et al. (2003) who suggested that CNG and  $Cl_{(Ca)}$  channels are closely arranged on the plasma membrane, although they do not constitute transduction complexes. If the CNG and  $Cl_{(Ca)}$  were to separate, internal diffusion of  $Ca^{2+}$  would be necessary to activate  $Cl_{(Ca)}$  channels, giving rise to a more complex transmission of information between these sequentially chained ion channels. The functions of  $Cl_{(Ca)}$  channel are, therefore, homogeneous along the cilium, similar to the CNG channels.

# 8.5.3 LINEAR SPATIAL SUMMATION OF LOCAL RESPONSES: HINDERED DIFFUSION OF CYTOPLASMIC FACTORS?

Takeuchi and Kurahashi (2008) showed that the cAMP responses induced by submicron local photolysis (Figure 8.1B) were independent within the cilium, especially when the response amplitude was small. Even within a 2 µm distance, double laser stimuli released within a short interval produced the same response amplitudes. This may indicate that the odorant-binding signal sums linearly, independent of the stimulus position and timing. Such linear summation of the local responses may suggest that the diffusion of cytoplasmic factors (cAMP and Ca<sup>2+</sup>) is hindered, when a limited number of molecules are produced locally. If cAMP and/or Ca<sup>2+</sup> could travel far from the site of photolysis, the adjacent response must be reduced by Ca<sup>2+</sup>-dependent adaptation. The diffusion of cytoplasmic factors within the thin cylindrical structure is obviously determined not only by their intrinsic diffusion properties, but also by buffering inside the cilium, and by extrusion/degradation systems. Furthermore, it should be noted that, in the ciliary nanostructure, the surface to volume ratio is extremely high and, therefore, soluble molecules have a high probability of interacting with molecules bound at the cytoplasmic membrane surface. Takeuchi and Kurahashi (2005) estimated that the maximum cytoplasmic cAMP level was of the order of  $100 \,\mu$ M, which is equivalent to ~500 molecules/µm-length (calculated from 0.1 µm diameter of the cilium). Since the density of the CNG channel is 300 channels/µm-length cilia (Kurahashi and Kaneko 1991), and each CNG channel has four cAMP binding sites (see e.g., Zheng and Zagotta 2004), the total number of cAMP binding sites

in 1 µm-length cilia is 1200. Therefore, the number of cAMP binding sites (1200) available is higher than the number of cAMP molecules produced by the odorant (500). Single CNG channels show open (bursting) events with a mean open time of ~100 ms, which is likely to represent the mean lifetime of the cAMP-CNG complex (Kurahashi and Kaneko 1993). Therefore, the CNG channels will initially trap most of the produced cAMP molecules with a very efficient surface to volume ratio, while some of them will be hydrolyzed by PDE. These processes may, at least in part, explain the outline of the hindered diffusion for the limited number of local cAMP molecules.

Because of the described properties of spatial linearity, ORC responses to small stimuli are additive, which increases the quantum efficiency of signal detection. At strong odorant stimulation, the length constant of the cilia becomes short and the total current reaches saturation before the CNG and  $Cl_{(Ca)}$  are fully activated. Due to such saturation, the ciliary membrane does not pass the ions at a distal area. This may help avoid Cl<sup>-</sup> depletion in the cilia.

### 8.5.4 CYCLIC NUCLEOTIDE-GATED CHANNELS: AN INITIAL POINT FOR SIGNAL AMPLIFICATION AND SITE OF SIGNAL MODULATION

As described in Section 8.5.1, CNG channels are involved in olfactory adaptation. A recent study indicates that CNG channels are also involved in the molecular mechanisms underlying olfactory masking. Indeed, Takeuchi et al. (2009) showed that not only can several types of odorants reduce the current through the CNG channel, but that the resulting odorant spectra show a positive correlation with olfactory masking measured in humans. Thus, at the olfactory cilia, both adaptation and masking are regulated at the level of CNG channels, although the mechanisms are different: negative feedback regulation in the case of adaptation and direct block by some odorants for masking. Interestingly, the  $Cl_{(Ca)}$  channel is not directly modulated in both phenomena (Boccaccio et al. 2006; Takeuchi et al. 2009), although a reduction in the CNG current causes a reduction of Ca<sup>2+</sup> influx, which, in turn, reduces the amplitude of  $Cl_{(Ca)}$  current.

It has been shown that the odorant- and cAMP-induced responses show similar cooperativities (see Figure 8.4C and E; Takeuchi and Kurahashi, 2005), indicating that a nonlinear amplification is established at a downstream point from cAMP production, due to the involvement of both CNG and  $Cl_{(Ca)}$  channels. Indeed, each channel is activated in a cooperative way and their sequential activation gives origin to a highly nonlinear amplification. Therefore, it is sufficient to modulate the primary CNG channel to affect adaptation and masking in a nonlinear way. In this respect, the modulation on CNG channels is expanded as an efficient effect at the transmission process (Figure 8.7). Furthermore, by directly modulating CNG instead of  $Cl_{(Ca)}$  channels,  $Ca^{2+}$  influx can be economically regulated. Since both channels are distributed evenly along the entire cilia, both signal generation and modulation are homogeneously regulated.

#### 8.6 CONCLUDING REMARKS

In this chapter, we have reviewed the basic principles of olfactory transduction in vertebrate olfactory cilia with a special emphasis on the functional role of CNG and  $Cl_{(Ca)}$  channels and their interaction with intracellular messengers. Although several components and modulators of the transduction cascade have been identified at the molecular level, many others, including  $Cl_{(Ca)}$  channels, some ion exchangers, and  $Ca^{2+}$ -binding proteins, remain to be identified. Their discovery will allow a better understanding of the transduction process occurring in our thin olfactory cilia.

#### ACKNOWLEDGMENTS

We thank Hiroko Takeuchi for interesting discussions. This work was supported by grants from: JSPS (T.K.), the Italian Ministry of Research (MIUR), and the Italian Institute of Technology (A.M.).

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# 9 Multiple Olfactory Subsystems Convey Various Sensory Signals

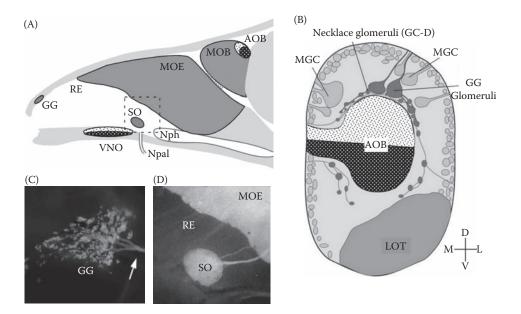
Minghong Ma

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# 9.1 INTRODUCTION

To detect a myriad of chemical cues signaling potential food, mates, and danger, most species (from worms, insects to mammals) develop sophisticated chemosensory systems. In mammals, the olfactory, gustatory, and trigeminal systems, which are primarily responsible for smell, taste, and somatosensation, respectively, are all involved in chemical senses. The nose, a seemingly unitary organ, consists of multiple olfactory apparatuses, among which the main olfactory epithelium (MOE) and the vomeronasal organ (VNO) have been extensively studied. Both systems comprise several subtypes of sensory cells with specialized morphological, molecular, and/or functional features. The MOE contains ciliated olfactory sensory neurons (OSNs) and microvillar cells. Most ciliated OSNs express G-protein-coupled odorant receptors (GPCRs) (Chapter 7) and employ the canonical cyclic adenosine monophosphate (cAMP) cascade to transform chemical energy into electrical signals



**FIGURE 9.1** The rodent nose contains multiple chemosensory systems. (A) Schematic illustration of the midsagittal view of the nasal cavity. The olfactory sensory neurons in the main olfactory epithelium (MOE) project to the main olfactory bulb (MOB). The apical and basal sensory neurons in the vomeronasal organ (VNO) send axons to the anterior and posterior accessory olfactory bulb (AOB), respectively. The septal organ (SO) (surrounded by the respiratory epithelium, RE) projects to the ventroposterior MOB, and the Grueneberg ganglion (GG) to the caudal MOB. Npal, nasopalatine duct; Nph, nasopharynx. The trigeminal system and the terminal nerve are not shown. (B) Organization of the caudal part of the olfactory bulb. MGC, modified glomerular complex; GC-D, guanylyl cyclase D; LOT, lateral olfactory tract; D, dorsal; M, medial; V, ventral; L, lateral. (C) The OMP-positive fluorescent neurons form the arrow-shaped GG in an OMP-GFP mouse. Individual cells project axons that fasciculate immediately to form a single nerve bundle (arrow). (D) The OMP-positive fluorescent neurons form the SO in an OMP-GFP mouse. The picture is taken from the dotted rectangle in (A). ([A] Modified from Ma M., *Crit. Rev. Biochem. Mol. Biol.*, 42, 463–80, 2007. [B, C] From Fuss S.H., Omura M., and Mombaerts P., *Eur. J. Neurosci.*, 22, 2649–54, 2005. With permission from Blackwell. [D] Adapted from Tian H. and Ma M., *Dev. Neurobiol.*, 68, 476–86, 2008b.)

(Chapter 8). Some of the ciliated OSNs express distinct chemoreceptors or noncanonical signal transduction machineries and project to specific regions in the olfactory bulb (OB) (see below). Likewise, the VNO contains at least two subsystems (the apical and basal compartments), which express two classes of vomeronasal receptors (V1Rs and V2Rs, respectively) and project to different portions of the accessory olfactory bulb (AOB) (Chapter 6). Additionally, some species (e.g., rodents) develop two spatially segregated clusters of sensory cells in the nasal cavity, forming the septal organ (SO) of Masera and the Grueneberg ganglion (GG) (Figure 9.1A). These chemosensory subsystems detect distinct but overlapping olfactory cues and some neurons may convey other sensory modalities transmitted by mechanical and thermal stimuli. This chapter covers several subsystems within the MOE as well as the SO and the GG. The key features of each subsystem will be discussed, including chemoreceptors, signal transduction cascades, central projections, and functional roles.

#### 9.2 SUBSYSTEMS WITHIN THE MAIN OLFACTORY EPITHELIUM (MOE)

The pseudostratified MOE contains heterogeneous populations of sensory cells, most with cilia but some with microvilla for sensing different signals. The ciliated, bipolar OSNs project their axons to the main olfactory bulb (MOB) and terminate in specific glomeruli based on their odorant receptor

(OR) identity. While the ciliated OSNs appear uniform in morphology, they can be divided into subpopulations that express distinct chemoreceptors or signal transduction machineries, including the canonical cAMP cascade, guanylyl cyclase type D (GC-D), trace amine-associated receptors (TAARs), and transient receptor potential channel M5 (TRPM5). The microvillar cells also consist of several subtypes, including the solitary epithelial cells expressing TRPM5 and the bipolar cells expressing another TRP channel, TRPC6. The subsystems utilizing the unconventional receptors or transduction pathways are the focus of this section.

#### 9.2.1 GUANYLYL CYCLASE TYPE D NEURONS

Guanylyl cyclases (membrane-bound or soluble) are a family of enzymes critically involved in transforming the external stimuli into intracellular signals in many cell types. Membrane-bound guanylyl cyclases are single transmembrane proteins with three functional domains: an extracellular receptor domain, an intracellular regulatory domain, and an intracellular catalytic domain that generates the second messenger, cyclic guanosine monophosphate (cGMP) (Garbers et al. 2006). One of the membrane-bound guanylyl cyclases, GC-D, was originally cloned from the rat olfactory epithelium (Fulle et al. 1995). GC-D-positive neurons are ciliated OSNs and scattered in the MOE (more concentrated in the recesses of the ectoturbinates rather than the endoturbinates) (Fulle et al. 1995; Juilfs et al. 1997; Walz et al. 2007) and the SO (Ma et al. 2003; Walz et al. 2007). These neurons clearly define a unique chemosensory subsystem in the MOE by their distinct signal transduction machineries and central targets in the OB. Unlike the majority of ciliated OSNs, GC-D neurons do not express the key elements in the cAMP-signaling pathway, such as  $G_{\text{group}}$  ACIII, CNGA2, PDE1C, and PDE4A (Chapter 8). Instead, they express a cGMP-stimulated phosphodiesterase (PDE2A) and a cGMP-specific CNG channel (CNGA3) in addition to GC-D (Fulle et al. 1995; Juilfs et al. 1997; Meyer et al. 2000). Although GC-D neurons are broadly distributed in the MOE, their axons coalesce onto a small number of "necklace" glomeruli, initially revealed by immunostaining of the axon terminals by a PDE2A antibody (Juilfs et al. 1997; Baker et al. 1999) and then confirmed in genetically labeled GC-D mice (Hu et al. 2007; Leinders-Zufall et al. 2007; Walz et al. 2007). These interconnected glomeruli form a ring that encircles the caudal end of the MOB and the anterior AOB (Figure 9.1B), a region that clearly contains heterogeneous glomeruli (Shinoda et al. 1989, 1993; Ring et al. 1997). Because some GG neurons also express PDE2A, it remains to be determined to what extent the GC-D neuron axons coalesce with the GG axons (Section 9.4).

Two potential functions have been proposed for GC-D neurons based on the identified chemosensory cues. First, GC-D neurons may serve as sensitive  $CO_2$  sensors.  $CO_2$  represents an important environmental cue that can be detected by many species, from worms (Hallem and Sternberg 2008), insects (Suh et al. 2004; Jones et al. 2007), to mammals (Youngentob et al. 1991; Hu et al. 2007). GC-D neurons coexpress carbonic anhydrase II (CAII), which catalyzes the rapid conversion of carbon dioxide to bicarbonate and proton. In behavioral tests, CAII null mice showed diminished responses to  $CO_2$  as compared with their wildtype counterparts. Individual GC-D neurons displayed excitatory responses to  $CO_2$ , which were blocked by carbonic anhydrase or CNG channel blockers. Furthermore, stimulation of the MOE by  $CO_2$  induced activity in the OB neurons associated with the necklace glomeruli (Hu et al. 2007). Further biochemical assays indicated that bicarbonate directly acts on the intracellular catalytic domain of GC-D to produce cGMP, which opens the CNG channel (Sun et al. 2009). Interestingly, the gene encoding GC-D becomes a pseudogene in many primates including humans (Young et al. 2007) and  $CO_2$  is odorless to humans.

Second, GC-D neurons may serve as specific detectors for natriuretic peptides and components of urine. Natriuretic peptides, a family of cGMP-regulating agonists, play an essential role in the maintenance of salt and water homeostasis (Forte 2004). GC-D neurons responded to two natriuretic peptides (uroguanylin and guanylin) by displaying an increased firing frequency and an elevated intracellular Ca<sup>2+</sup> level, and the responses were eliminated in knockout mice lacking GC-D (Leinders-Zufall et al. 2007). In electro-olfactogram (EOG) recordings, the MOE responded to uroguanylin and guanylin with high sensitivity, and the responses were retained in CNGA2<sup>-/-</sup> but not in CNGA3<sup>-/-</sup> mice, supporting the involvement of a cGMP cascade (Leinders-Zufall et al. 2007). The peptide receptors in GC-D neurons are still elusive, but GC-D itself serves as a good candidate because it shares a similar ligand-binding domain as other guanylyl cyclases responding to natriuretic peptides (Forte 2004). Consistent with this notion, uroguanylin (but not guanylin) stimulates rat GC-D in heterologous cells (Duda and Sharma 2008). Since individual GC-D neurons showed different tuning properties, i.e., some responded to both peptides and others responded to only one (Leinders-Zufall et al. 2007), it is possible that some unidentified chemoreceptors are coexpressed in GC-D neurons and are involved in peptide detection. It is possible that detection of  $CO_2$  and peptide signals by GC-D neurons is integrated at the cellular and behavioral level, but the underlying mechanisms and functional implications remain elusive.

#### 9.2.2 TRACE AMINE-ASSOCIATED RECEPTOR (TAAR) EXPRESSING NEURONS

The main olfactory system is conventionally thought to detect volatile odors only, but several studies indicate that it also responds to social cues carried via volatile pheromones and small peptides (Lin et al. 2004, 2005; Xu et al. 2005; Spehr et al. 2006; Wang et al. 2006). This raises the possibility that OSNs in the MOE may express chemoreceptors other than GPCRs (Chapter 7). In a large-scale search for additional GPCRs expressed in the olfactory epithelium, Liberles and Buck (2006) discovered a second class of chemosensory receptors, the TAARs. Originally identified in the brain as amine receptors (Borowsky et al. 2001), TAARs share some sequence similarities with receptors of classical biogenic neurotransmitters, such as serotonin and dopamine, but not with ORs. The TAAR family is found in all vertebrates, including fish (>100 members in zebrafish), amphibian (six members in frog), rodent (15 members in mouse), and human (five members) (Gloriam et al. 2005; Liberles and Buck 2006; Hashiguchi and Nishida 2007). The mouse TAARs are divided into nine subtypes (TAAR1 to 9) with each subtype containing one member, except TAAR7 (five highly related members) and TAAR8 (three members). Eight out of the nine subtypes (TAAR2–9) were primarily found in the olfactory epithelium in similar patterns as ORs, i.e., each TAAR member is detected in a small subset of OSNs scattered in discrete zones and is not coexpressed with other TAARs or ORs. Some TAAR members are expressed in the GG neurons (Section 9.4.3). The central targets of the TAAR-expressing OSNs have not been determined. It is not clear whether the OSNs expressing the same TAAR converge their axons onto specific glomeruli. The TAAR-positive OSNs in the MOE coexpress  $G_{colf}$ , suggesting that these receptors transduce signals via the canonical cAMP pathway. However, this requires verification by direct measurement of the responses from TAAR-expressing OSNs, which has not been achieved. In a heterologous expression system, interestingly but not surprisingly, several TAAR members were found to detect specific amines, including three that are present in the urine. One member (TAAR5) was activated by diluted urine from sexually mature males only, but not from females or prepubescent males (Liberles and Buck 2006). While some TAAR-expressing OSNs may be involved in detecting species-specific social cues, these chemoreceptors are likely to serve more general functions since TAARs are conserved across many species. Further studies on the central targets of these neurons and the behavioral deficits of knocking out these genes will help to unveil the specific function this subsystem serves.

#### 9.2.3 TRANSIENT RECEPTOR POTENTIAL CHANNEL EXPRESSING CELLS

The TRP ion channels are involved in mediating the electrical signals in sensory cells for different modalities, including touch, pain, temperature, sound, pheromones, and taste. The TRP proteins are six-transmembrane (6-TM) cation-permeable channels and opening of these channels leads to depolarization of the cell membrane. On the basis of sequence homology, mammalian TRP proteins can be grouped into six subfamilies: TRPC, TRPV, TRPM, TRPA, TRPP, and TRPML, which are

activated by diverse mechanisms (Ramsey et al. 2006; Venkatachalam and Montell 2007). Several TRP channels are present in distinct subpopulations of sensory cells in the nasal cavity.

# 9.2.3.1 Transient Receptor Potential Channel M5-Positive Cells

TRPM5 was originally identified in taste receptor cells (Perez et al. 2002; Zhang et al. 2003). In a TRPM5-GFP transgenic line (GFP expression is driven by the TRPM5 promoter), surprisingly, GFP cells are found in the nasal epithelium. There are at least two subtypes of TRPM5-expressing cells: a large population of ciliated OSNs and a subset of solitary microvillar cells (Lin et al. 2007, 2008).

TRPM5-positive OSNs are predominantly located in the ventrolateral areas of the olfactory epithelium and project to the ventral regions of the OB. These neurons are positively stained by antibodies against phospholipase C (PLC)  $\beta$ 2 and G protein  $\gamma$ 13, two components that are involved in taste transduction in addition to TRPM5 (Huang et al. 1999; Perez et al. 2002; Zhang et al. 2003). Most TRPM5 neurons also express CNGA2, suggesting the coexistence of the cAMP and the phosphoinositide (PI) signaling pathways in individual OSNs (Lin et al. 2007). These neurons are probably involved in the detection of semiochemicals, because the periglomerular cells associated with TRPM5-positive glomeruli are activated by mouse urine and putative pheromones (Lin et al. 2007). With an intact cAMP-signaling cascade, the MOE of TRPM5 null mice did not show reduced odor or pheromone responses in EOG recordings. However, in CNGA2 knockout mice or in the presence of an adenylyl cyclase inhibitor, the PI pathway might mediate the residual odor or pheromoneinduced responses (Lin et al. 2004, 2007). TRPM5-expressing neurons represent a subset of OSNs that may detect general odors as well as pheromones, using a dual signal transduction cascade.

TRPM5 is also found in a subset of solitary microvillar cells, which are especially concentrated in the anterior part of the respiratory epithelium in the nasal cavity (Kaske et al. 2007; Lin et al. 2007, 2008). These epithelial cells were originally characterized by the expression of T2R "bittertaste" receptors,  $\alpha$ -gustducin, and PLC $\beta$ 2 (Finger et al. 2003), and TRPM5 probably serves as a downstream channel in this signal transduction pathway. However, TRPM5-expressing cells do not always express these taste signal transduction machineries, indicating these cells consist of different subpopulations (Lin et al. 2008). These cells form synaptic-like contacts with trigeminal afferent nerve fibers (Finger et al. 2003; Lin et al. 2008), which carry the sensory information into the brain. Irritating odorants at relatively high concentrations induced electrical signals in the anterior respiratory epithelium and caused Ca<sup>2+</sup> elevation in dissociated TRPM5-positive cells (Lin et al. 2008). These solitary chemosensory cells are probably involved in sensing harmful or irritant chemicals that trigger protective reflexes (such as sneezing and apnea) mediated by the trigeminal system.

# 9.2.3.2 Transient Receptor Potential Channel 6-Positive Cells

TRPC6 is expressed in a distinct subtype of microvillar cells in the MOE. Unlike ciliated OSNs, TRPC6<sup>+</sup> cells do not express olfactory marker protein (OMP) or the key elements in the canonical cAMP cascade. Instead, they express PLC  $\beta$ 2, TRPC6, and inositol 3,4,5-trisphosphate receptors type III (InsP3R-III) (Elsaesser et al. 2005). These bipolar cells express neuronal marker proteins and possess an axonlike process, which does not extend through the basal lamina. This is consistent with the fact that these cells do not degenerate following bulbectomy, suggesting a local role in chemoreception within the MOE. The chemoreceptors expressed in these cells are undetermined, even though they responded to some odorants by showing an elevated Ca<sup>2+</sup> level (Elsaesser et al. 2005). Interestingly, some TRPC6<sup>+</sup> cells also express neuropeptide Y, which suggests that they may play a role in the development and/or regeneration of the olfactory epithelium (Montani et al. 2006).

# 9.2.3.3 Transient Receptor Potential Channel 2-Positive Cells

TRPC2 is a key element of the signal transduction cascade in the VNO neurons. While a TRPC2 antisense probe strongly labels the VNO neurons, it faintly stains a small subset of cells in the adult

and embryonic MOE. These TRPC2<sup>+</sup> cells are probably immature neurons because of their basal location, and their function is unknown (Liman et al. 1999).

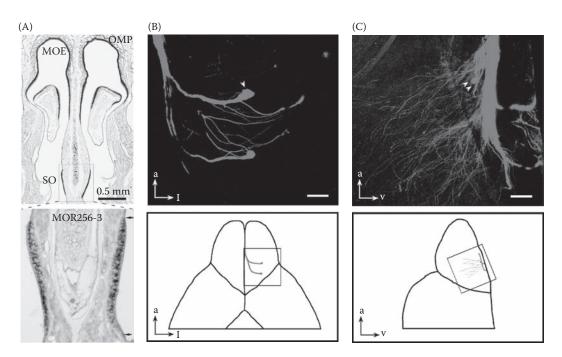
#### 9.3 THE SEPTAL ORGAN (SO)

The SO (also termed Organ of Masera) is a small island of olfactory neuroepithelium lying bilaterally at the ventral base of the nasal septum near the entrance of the nasopharynx (Figure 9.1A and D). It was first observed in newborn mice (Broman 1921) and subsequently described in detail by Rodolfo-Masera (1943). This olfactory apparatus has been observed in many mammals, including rat, mouse, hamster, deer mouse, rabbit, opossum, guinea pig, bandicoot, and koala (Rodolfo-Masera 1943; Adams and McFarland 1971; Bojsen-Moller 1975; Katz and Merzel 1977; Breipohl et al. 1983, 1989; Kratzing 1984a, 1984b; Taniguchi et al. 1993), but not in cat (Breipohl et al. 1983) or ferret (Weiler and Farbman 2003). The SO resembles the MOE in cellular composition and is composed of ciliated OSNs, microvillar cells, supporting cells, and Bowman's glands (Graziadei 1977; Miragall et al. 1984; Kratzing 1984a, 1984b; Breipohl et al. 1989; Adams 1992; Taniguchi et al. 1993; Giannetti et al. 1995a). However, some morphological differences are observed between these two systems. The SO contains fewer layers of OSNs (two to three layers) as compared to the MOE (six to eight layers in most regions) (Ma et al. 2003; Weiler and Farbman 2003). Furthermore, the SO OSNs have flattened somata, shortened dendrites, and larger dendritic knobs, representing one of the rare differences described among the otherwise uniform morphology of ciliated OSNs in the MOE (Ma et al. 2003).

#### 9.3.1 ODORANT RECEPTORS AND SIGNAL TRANSDUCTION

The chemoreceptors expressed in the SO have been described in great detail. Using different degenerate primers in a cDNA cloning approach, two groups have collectively detected more than 120 candidate OR genes in the mouse SO (Kaluza et al. 2004; Tian and Ma 2004). However, the expression levels of individual OR genes vary dramatically, verified by Affymetrix genechips covering all the mouse olfactory receptor genes (a high-density oligonucleotide array suitable for monitoring the expression of a large number of genes simultaneously) (Zhang et al. 2004) and in situ hybridization (a method of detecting transcribed mRNAs of certain genes by specific antisense RNA probes). The SO of young adult mice mainly expresses a few ORs from a repertoire of ~1000, with the most predominant OR (MOR256-3, also known as SR1 or Olfr124) in nearly 50% of the cells (Figure 9.2A) and the nine most predominant ORs (MOR256-3, 244-3, 235-1, 0-2, 256-17, 236-1, 160-5, 122-1, and 267-16) together in ~95% of the cells. Notably, the cells expressing different ORs have asynchronous temporal onsets and differential growth rates during pre- and postnatal development (Tian and Ma 2008b). The unusually high density of MOR256-3 cells in the SO raises the question of whether a single cell expresses a single OR type. Experiments with combined OR probes in double *in situ* hybridization reiterate the one cell-one receptor tenet with few exceptions. Interestingly, the coexpression frequency of MOR256-3 vs a mixture of the remaining eight ORs in single neurons is nearly ten times higher in newborn mice (2.0% at P0 vs 0.2% at postnatal four weeks) or following four-week sensory deprivation. The olfactory epithelium seems to utilize an activity-dependent mechanism to ensure the singular expression pattern in a subset of OSNs (Tian and Ma 2008a). All nine abundant SO ORs are also expressed in the most ventrolateral zone of the MOE, even though the relative abundance does not match that in the SO (Tian and Ma 2004).

Most SO neurons express  $G_{\alpha olf}$  and ACIII, suggesting that signal transduction in these neurons is mediated by the canonical cAMP pathway (Chapter 8), which is further supported by patch-clamp recordings from individual OSNs in this region. An adenylyl cyclase activator and a phosphodiesterase inhibitor mimicked odorant responses, and these responses were blocked by an adenylyl cyclase inhibitor and eliminated in CNGA2 null mice (Ma et al. 2003; Grosmaitre et al. 2007).



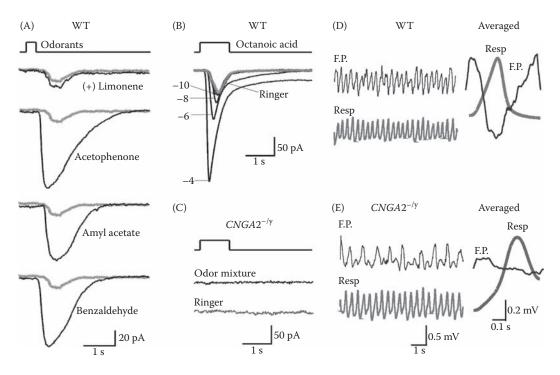
**FIGURE 9.2** The Fseptal organ predominantly expresses a few odorant receptors and projects to the main olfactory bulb. (A) Two coronal sections were hybridized with an OMP (upper panel) or an MOR256-3 (lower panel) antisense RNA probe. Approximately 50% of septal organ neurons from adult animals express MOR256-3. (B, C) DiI-labeled SO fibers enter the bulb on the medial side and terminate in a few "septal" glomeruli (arrowheads) and many other glomeruli in the ventral (B) and medial (C) view. The lower panels illustrate the location that the DiI fibers innervate. Scale bars=200 µm. a, anterior; l, lateral; v, ventral. ([A] Upper panel adapted from Tian H. and Ma M., *J. Neurosci.*, 24, 8383–90, 2004. Lower panel from Tian H. and Ma M., *J. Neurosci.*, 24, 8383–90, 2004. Lower panel from Tian H. and Ma M., *J. Neurosci.*, 120, 483–92, 2003. With permission from Springer Science+Business Media.)

#### 9.3.2 CENTRAL PROJECTION

SO neurons send off a few axon bundles, which travel across the cribriform plate and terminate in the MOB. In rats, focal injection of horseradish peroxidase (HRP) in the MOB resulted in labeled cells in the SO (Pedersen and Benson 1986), while retrograde injection of HRP in the SO traced the axons to the posterior, ventromedial OB with a few densely labeled and many lightly labeled glomeruli (Astic and Saucier 1988; Giannetti et al. 1992). More recently, neurotracing studies using lipophilic dyes, such as DiI, were carried out in the mouse SO (Levai and Strotmann 2003; Ma et al. 2003). Genetically targeted OMP-GFP mice (the coding region of OMP was replaced by tauGFP; Potter et al. 2001) allow accurate identification and thus precise placement of DiI onto this area (Figure 9.1D) (Levai and Strotmann 2003). The labeled axons projected mainly to the posterior, ventromedial OB and targeted onto a few densely labeled glomeruli (so called "septal" glomeruli) receive inputs mainly (if not exclusively) from the SO, while the lightly labeled glomeruli receive mixed inputs from the SO and the MOE. The projection pattern of the SO is consistent with the fact that this region expresses a few ORs at high densities and many other ORs sparsely.

#### 9.3.3 BROAD RESPONSIVENESS AND MECHANOSENSITIVITY

Although the SO primarily covers a small fraction of the receptor repertoire, the majority (~70%) of SO neurons were surprisingly responsive to diverse chemicals with different size, shape, and



**FIGURE 9.3** Most septal organ neurons respond to a wide range of odorants and to mechanical stimulation. (A) A single neuron from a wildtype (WT) animal responded to multiple odorants (except (+) limonene) at 300  $\mu$ M, recorded with perforated patch-clamp. Inward currents were elicited by odor and Ringer puffs under voltage-clamp mode. (B) A single neuron responded to octanoic acid puffs at different concentrations (10<sup>-10</sup> to 10<sup>-4</sup> M) under voltage-clamp mode. The gray trace indicates the response induced by puffing Ringer. (C) Both odorant and mechanical responses were eliminated in CNGA2<sup>-/y</sup> mice. The holding potential for all neurons was –60 mV. (D, E) Oscillatory field potentials (F.P., black) in the olfactory bulb were recorded (at a depth of 100  $\mu$ m from the surface) in wildtype (WT) (C) or CNGA2<sup>-/y</sup> mice (D). Traces marked with Resp (gray) indicate the respiratory rhythm. The averaged field potential within one respiratory cycle is shown in the right column of each panel. (Modified from Grosmaitre X. et al. *Nat. Neurosci.*, 10, 348–54, 2007.)

functional groups, as demonstrated by patch-clamp recordings (Figure 9.3A). These neurons were extremely sensitive to some odorants with a nanomolar threshold and a wide dynamic range, which covers 3–4 log units of concentration from threshold to saturation (Figure 9.3B) (Grosmaitre et al. 2007). This is consistent with a previous EOG study in which the SO responded to many chemicals with a lower threshold than the MOE (Marshall and Maruniak 1986).

The most dominant receptor, MOR256-3, was then confirmed to confer broad tuning to mouse OSNs using genetically targeted mouse lines. All OSNs expressing this receptor (regardless of their location in the SO or in the MOE) were highly responsive, while genetic deletion of this receptor resulted in more selective OSNs. The broad response spectrum of MOE256-3 was also verified in a heterologous cell line (our unpublished data). The SO, situated in the direct air path, may thus serve as a general odor detector and/or a sensor of the total odor concentration to "alert" the organism, a function originally proposed by Rodolfo-Masera (1943). The potential alerting role was not verified in a behavioral study with surgical removal of the SO (Giannetti et al. 1995b), probably due to the existence of similar OSNs in the MOE. An alternative and complementary hypothesis was suggested: that the SO functions as a "mini-nose" in surveying food odors as well as social cues (Breer et al. 2006).

Another surprising finding that arises from the SO is that many neurons responded not only to odorants, but also to mechanical stimuli delivered by pressure ejections of odorant-free Ringer solution (Figure 9.3A and B). The mechanical responses directly correlated with the pressure intensity and similar mechanosensitivity also existed in ~50% of the neurons in the MOE. The responses occurred with relatively long delays and were completely blocked by an adenylyl cyclase inhibitor, suggesting the involvement of cAMP as a second messenger. Elimination of mechanosensitivity in the OSNs from CNGA2 knockout mice further supports this notion (Figure 9.3C). Thus, the chemical and mechanical responses of the OSNs are mediated by a shared cascade involving cAMP and the CNG channel (Grosmaitre et al. 2007). The mechanosensitivity is probably tied to certain OR types, because all OSNs expressing MOR256-3 showed mechanical responses, while most OSNs with a deleted MOR256-3 gene displayed no mechanosensitivity (Grosmaitre et al. 2009). The SO can thus serve as an airflow sensor in addition to its chemosensory roles.

The mechanosensitivity found in the OSNs is particularly interesting, because these neurons are situated in the nostril and constantly experience episodic pressure changes carried by the airflow. One possible role of the mechanosensitivity is that when the air flows faster in the nose, such as during a powerful sniff, it can enhance the firing probability and frequency of individual OSNs weakly stimulated by odorants. In addition to the critical roles in odor delivery and sampling (Verhagen et al. 2007), sniffing may increase the overall sensitivity of the olfactory system via the mechanosensitivity of the sensory neurons (Grosmaitre et al. 2007). A second role that the mechanosensitivity of the OSNs may play is to synchronize the rhythmic activity (theta-band oscillation) in the OB with the breathing cycles, even in the absence of odorants, priming the system for processing odor information. In CNGA2 null mice, the OSNs failed to exhibit odorant and mechanical responses (Figure 9.3C), and the coupling between the bulb rhythmic activity and respiration was drastically reduced (Figure 9.3D and E). Therefore, the mechanosensitivity of the OSNs, in addition to episodic access to odorants, may cause the respiration-coupled, odorant-induced activity in the olfactory epithelium (Chaput 2000), the OB (Adrian 1951; Ueki and Domino 1961; Macrides and Chorover 1972; Onoda and Mori 1980; Philpot et al. 1997; Luo and Katz 2001; Cang and Isaacson 2003; Spors et al. 2006), and the olfactory cortex (Fontanini et al. 2003; Rennaker et al. 2007). The mechanosensitivity of OSNs provides new insights into the important roles played by sniffing in olfactory perception (Mainland and Sobel 2006). In fact, sniffing alone is sufficient to induce activities in the olfactory cortex in human subjects (Sobel et al. 1998). It remains to be determined how the "odor maps" along the olfactory pathway are modified under different breathing and sniffing patterns.

#### 9.4 THE GRUENEBERG GANGLION (GG)

#### 9.4.1 CENTRAL TARGETS

The GG is located bilaterally to the anterior vestibule of the nasal cavity (Figure 9.1A). Unlike the pseudostratified MOE, VNO, and SO, each GG contains small grape-like clusters of ~500 neurons (Figure 9.1C). This organ was found in all mammals examined, including human, and was originally thought to be nonsensory and part of the terminal nerve system (Grueneberg 1973). However, recent studies rediscovered the GG as a chemosensory organ. Surprisingly, GG neurons express OMP (a specific marker for mature chemosensory neurons) and, consequently, they are readily visible in genetically targeted OMP-GFP mice (Figure 9.1C). The GG starts to form around embryonic day 16 and becomes fully developed at birth. The axons of GG neurons fasciculate into a single or a few nerve bundles and project to the caudal MOB (Fuss et al. 2005; Koos and Fraser 2005; Fleischer et al. 2006a; Roppolo et al. 2006; Storan and Key 2006). Neurotracing by DiI reveals that GG axons innervate ~10 glomeruli (including one or two large ones) in the same region as the necklace glomeruli (the central targets of GC-D neurons) (Figure 9.1B). Because a subset of GG neurons (V2r83-positive, see below) express PDE2A, but not GC-D or CAII (two specific proteins in GC-D neurons) (Fuss et al. 2005; Fleischer et al. 2005; Fleischer et al. 2005; Storan in protein apparently contain heterogeneous populations. It is possible that individual PDE2A glomeruli converge

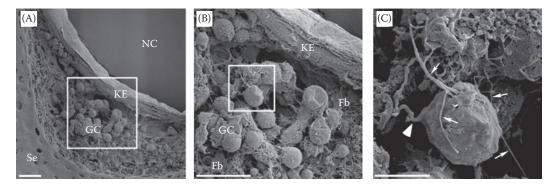
inputs from GC-D and GG neurons, since all PDE2A glomeruli are innervated by GC-D neurons (Leinders-Zufall et al. 2007). However, the central targets of these two systems should be segregated at least to some extent, because some necklace glomeruli are homogenously innervated by GC-D fibers (Walz et al. 2007). Similar to the sensory neurons in other nasal organs, GG neurons undergo degeneration after axotomy, suggesting that the survival of these neurons depends on their central connections (Roppolo et al. 2006; Brechbuhl et al. 2008).

## 9.4.2 MORPHOLOGY OF GRUENEBERG GANGLION (GG) NEURONS

GG neurons appear to lack the typical chemoreceptive structures, such as cilia or microvilli, under light or confocal microscopy (Fuss et al. 2005; Fleischer et al. 2006a; Roppolo et al. 2006; Storan and Key 2006). Using scanning electron microscopy, a recent study demonstrates that clusters of GG cells are located in a fibroblast meshwork between the nasal septum and a keratinized epithe-lium (KE) (Figure 9.4). Further examination by transmission electron microscopy reveals that each GG cell possesses 30–40 nonmotile primary cilia (15  $\mu$ m long and 0.2  $\mu$ m thick). These cilia are profoundly invaginated into the cytoplasm and are found in the extracellular matrix. Moreover, the GG also contains glial cells (immunostained by glial markers), which wrap around the OMP-positive neurons and trap most cilia within the ganglion. Since the cilia of GG neurons do not cross the KE layer to reach the nasal cavity, it raises the question of whether chemical compounds can reach these neurons. In standard skin permeability assays, the KE appears to be leaky, suggesting that water-soluble chemicals can have access to GG neurons (Brechbuhl et al. 2008).

## 9.4.3 CHEMORECEPTORS AND SIGNAL TRANSDUCTION

Identification of chemoreceptors in GG neurons further supports their role in chemoreception. By combining RT-PCR and in situ hybridization, a substantial portion of OMP-positive GG neurons is found to express V2r83 (or V2R2), a V2R subtype expressed in all basal VNO neurons (Fleischer et al. 2006b). The remaining OMP-positive, V2r83-negative cells express several subtypes of TAARs (Fleischer et al. 2007). Certain ORs are transiently expressed in the GG. For instance, MOR256-17 is expressed in very few cells at the embryonic stages, but its expression disappears at postnatal stages (Fleischer et al. 2006b). Some signal transduction elements are identified in GG neurons, including  $G\alpha_0$  and  $G\alpha_{12}$ , which are probably coexpressed in single neurons (Fleischer



**FIGURE 9.4** GG neurons contain ciliary processes. (A) A scanning EM micrograph shows a GG coronal section. Clusters of GG cells (GC) are located along the nasal septum (Se) underneath the keratinized epithelium (KE). NC, nasal cavity. Square detailed in (B). (B) An enlarged view of the GG cells in a fibroblast meshwork (Fb). Square detailed in (C). (C) A GG cell with its axon (arrowhead) and thin ciliary processes (arrows). Scale bar=20  $\mu$ m in (A, B) and 5  $\mu$ m in (C). (Adapted from Brechbuhl J., Klaey M., and Broillet M.C., *Science*, 321, 1092–95, 2008. With permission from AAAS.)

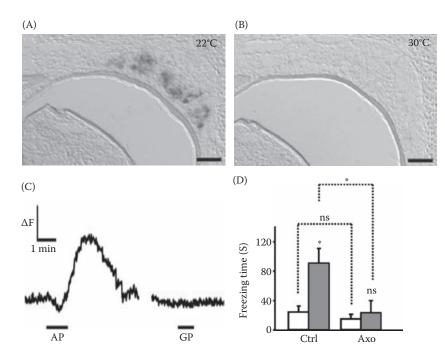
et al. 2006b). Interestingly, the V2r83-positive neurons coexpress a transmembrane guanylyl cyclase subtype (GC-G) and PDE2A (Fleischer et al. 2008), indicating the existence of a cGMP-mediated signal transduction pathway.

#### 9.4.4 FUNCTIONS

#### 9.4.4.1 Sensing Coolness

The number of GG neurons is particularly high in perinatal stages, followed by a decline in postnatal development, suggesting a more important function of this organ in newborns (Fuss et al. 2005; Fleischer et al. 2007). A subset of the necklace glomeruli (MGC: modified glomerular complex, Figure 9.1B) in rodent pups is activated during suckling behavior (Teicher et al. 1980; Greer et al. 1982), which leads to the hypothesis that the GG may play a role in mother/child interaction. To test this possibility, Mamasuew et al. (2008) examined c-Fos expression in the GG neurons of neonatal mouse pups in the presence and absence of the dam. Surprisingly, these neurons were only activated in the absence of the mother, and the activation was independent on olfactory cues revealed by naris closure. Cool ambient temperatures (but not warmer temperatures) were then confirmed to induce strong activity in V2r83-positive GG neurons (Figure 9.5A and B). The responses were significantly reduced in older stages, suggesting that these GG neurons serve as a thermosensor in newborns.

V2r83-positive GG neurons do not express TRPM8 (Fleischer et al. 2009), an ion channel essential for cold thermosensation (Bautista et al. 2007). These neurons instead coexpress several key components of the cGMP cascade (such as GC-G and PDE2A) (Fleischer et al. 2009), similar to



**FIGURE 9.5** The Grueneberg ganglion fulfils multiple functions. (A, B) Placing isolated pups at 22°C (A) but not at 30°C (B) for 3 h induced c-Fos expression in the GG neurons. (C) Alarm pheromones (AP), but not general pheromones (GP), induced a reversible calcium transient in GG neurons. (D) APs in artificial cerebrospinal fluid (ACSF) solution (white bars, ACSF; gray bars, ACSF+AP) induced a significant increase in freezing behavior in control (Ctrl), but not in GG axotomized (Axo) OMP-GFP mice. ([A, B] Adapted from Mamasuew K., Breer H., and Fleischer J., *Eur. J. Neurosci.*, 28, 1775–85, 2008. With permission from Blackwell. [C, D] Adapted from Brechbuhl J., Klaey M., and Broillet M.C., *Science*, 321, 1092–95, 2008. With permission from AAAS.)

the chemo- and thermosensitive AWC neurons in *Caenorhabditis elegans* (Kuhara et al. 2008). The molecular mechanisms underlying thermosensing in GG neurons remain to be determined.

#### 9.4.4.2 Sensing Alarm Signals

Another group extensively researched the chemostimulus for GG neurons using calcium imaging on coronal tissue slices (Brechbuhl et al. 2008). A variety of stimuli, including mouse milk, mammary secretions from lactating female mice, a mix of odorants, some known pheromones, mouse urine, and  $CO_2$ , did not enhance the fluorescence of the GG neurons. Strikingly, alarm pheromones collected during the killing of mice with  $CO_2$  induced transient calcium signals in almost all GG neurons from newborn and adult mice (Figure 9.5C). The calcium ions were preferentially released from the internal stores, because the signals were present in divalent-free solutions, but abolished by depletion of the internal calcium stores. The identity of the effective compounds in the alarm pheromones is still elusive.

The same group also examined the behavioral relevance of alarm pheromone sensing via GG cells by sectioning the GG axon bundles. Alarm pheromones elicited a stereotyped freezing reaction in rodents (Kikusui et al. 2001). Control mice displayed similar freezing behavior after exposure to alarm pheromones, which induced calcium signals in GG neurons. However, after the GG axotomy, the freezing behavior was replaced by exploring activity (Figure 9.5D). The behaviors of these mice were no longer affected by the same alarm pheromones. These results strongly support a role for GG neurons in alarm sensing. The GG apparently serves multiple functions and it is not clear if coolness and alarm signals are detected by the same set of GG neurons. Humans also possess this organ (Grueneberg 1973), but whether it has the same function(s) is not clear.

#### 9.5 CONCLUDING REMARKS

Organization of the mammalian nose is more complicated than previously appreciated. Each of the four physically segregated apparatuses (MOE, VNO, SO, and GG) contains heterogeneous cell types with distinct chemoreceptors, transduction machineries, and/or central targets. Each organ can convey sensory information of multiple modalities and serve multiple functions. More subsystems may still emerge with new molecular markers and more detailed anatomical/functional analysis. Cutting-edge technology applied in modern neuroscience from various disciplines holds great hopes for revealing the specific roles played by each subsystem.

The advantage of having multiple olfactory subsystems is manifold. The different chemoreceptors expressed in these subsystems can expand the overall detection capacity of the olfactory system for chemicals and other stimulations. In addition, critical information can be processed in parallel by multiple subsystems, which send signals to different brain regions for further integration and execution. The diversity and complexity of the chemosensory systems allow the organisms to accurately perceive their chemical surroundings and respond appropriately by adjusting their behaviors, emotions, and hormones.

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# 10 Feedback Regulation of Neurogenesis in the Mammalian Olfactory Epithelium: New Insights from Genetics and Systems Biology

Kimberly K. Gokoffski, Shimako Kawauchi, Hsiao-Huei Wu, Rosaysela Santos, Piper L.W. Hollenbeck, Arthur D. Lander, and Anne L. Calof

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#### **10.1 INTRODUCTION**

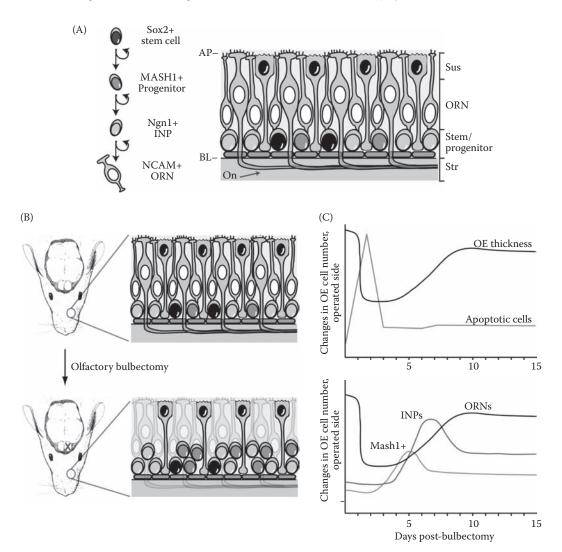
#### 10.1.1 ONGOING NEUROGENESIS IN THE OLFACTORY EPITHELIUM (OE) IN VIVO AND IN VITRO

The mouse olfactory epithelium (OE) is an ideal model system for identifying and characterizing the factors that regulate proliferation and differentiation of neurons from their stem and progenitor cells. In part, this is because the OE undergoes neurogenesis throughout life, and does so exuberantly in response to injury (Graziadei and Monti Graziadei 1978; Mackay-Sim and Kittel 1991; Calof et al. 2002). However, another advantage of great significance is the fact that numerous studies have given us a good idea of the cell types that give rise to olfactory receptor neurons (ORNs) (Cau et al. 1997; Calof et al. 2002; Kawauchi et al. 2004, 2005; Beites et al. 2005; see also Chapter 5). Thus, in the neuronal lineage of the OE, four cell stages have been identified, in vitro and in vivo: (1) Sox2-expressing stem cells, which reside in the basal compartment of the epithelium, are thought to commit to the ORN lineage via expression of the proneural gene, Mash1. (2) Mash1-expressing early progenitor cells, which divide and may act as transit-amplifying cells (Gordon et al. 1995), in turn give rise to (3) late-stage transit-amplifying cells, also known as immediate neuronal precursors (INPs), which express a second proneural gene, Ngn1 (Wu et al. 2003). INPs give rise to daughter cells that undergo terminal differentiation into (4) postmitotic Ncam-expressing ORNs. Figure 10.1A shows schematics of both the OE neuronal lineage and the spatial distribution of these cells within the OE in vivo. As is common to many epithelia, differentiation in the OE proceeds in a basal-to-apical direction: dividing stem and progenitor cells lie atop the basal lamina, and multiple layers of differentiated ORNs lie above the progenitor cells layers.

Since the OE is able to sustain de novo neurogenesis throughout life and to regenerate in response to injury (Graziadei and Monti Graziadei 1978; Calof et al. 2002), it must contain stem cells. Indeed, several groups have been interested in harvesting OE stem cells for their therapeutic potential (e.g., Zhang et al. 2004; Othman et al. 2005). However, when OE is isolated and cultured in serum-free medium, although it avidly generates neurons for one to two days (Calof and Chikaraishi 1989), it rapidly loses the ability to undergo neurogenesis unless other factors or feeder cells are added (DeHamer et al. 1994; Holcomb et al. 1995; Mumm et al. 1996; Shou et al. 2000). In other words, OE neuronal stem and transit-amplifying cells in isolation are prone to undergoing differentiative divisions over self-replicative divisions, resulting in rapid expiration of these cell populations in tissue culture. This observation has prompted numerous studies to search for the environmental cues that are important for sustaining stem and progenitor cell self-renewal and maintaining the neurogenic potential of the OE.

#### 10.1.2 REGENERATION IN THE OLFACTORY EPITHELIUM (OE) FOLLOWS A SPECIFIC TIME COURSE

Ultimately, regeneration in the OE is a mechanism for producing neurons when neurons are lost. Several injury models have been used to study neuronal regeneration in the OE. One of these, methyl bromide inhalation, in which exposure to methyl bromide gas damages all cell types in the OE and adjacent respiratory epithelium nonselectively, is not considered in this chapter (for details, see



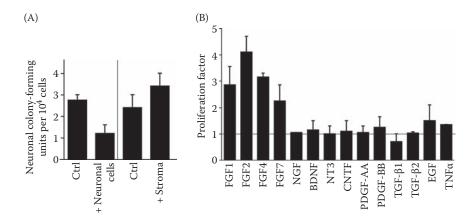
**FIGURE 10.1** Schematic of changes in OE cell populations as a result of olfactory bulbectomy over time. (A) Diagram of ORN lineage and cell lamination in the mature OE. From apical (Ap) to basal: Sus=sustentacular cells (glial cells), with somata adjacent to the nasal cavity; ORN=olfactory receptor neuron layers, containing NCAM+ ORNs; stem/progenitor cell layers, including horizontal basal cells adjacent to the basal lamina (BL), Sox2+ stem cells, Mash1+ early progenitor cells, and Ngn1+ immediate neuronal precursor (INP) cells; Str=stroma; On=olfactory nerve (ORN axons). (B, C) Removal of one olfactory bulb (olfactory bulbectomy) leads to rapid apoptosis of ORNs and a subsequent increase in progenitor cell numbers (MASH1+ cells followed by INPs) in the ipsilateral OE. As new ORNs are generated, progenitor cell numbers decrease until a new steady state is restored. Charts are drawn as relative changes in cell numbers in the OE ipsilateral to OB removal. (Values taken from Schwartz Levey, M., Chikaraishi, D.M., and Kauer, J.S., *J. Neurosci.*, 11, 3556–64, 1991; Gordon, M.K. et al. *Mol. Cell Neurosci.*, 6, 363–79, 1995; Holcomb, J.D., Mumm, J.S., and Calof, A.L., *Dev. Biol.*, 172, 307–23, 1995. With permission.)

Schwob et al. 1995, 2002; Huard et al. 1998; Jang et al. 2003). Probably the most selective surgical procedure for inducing neuronal degeneration and subsequent neurogenesis in the OE of rodents is surgical removal of one of the two olfactory bulbs (OBs) of the brain (unilateral "bulbectomy," schematized in Figure 10.1B; since the OBs are the direct synaptic targets of ORNs, bulbectomy severs ORN axons). Unilateral bulbectomy causes a selective degeneration of ORNs, and numerous

studies have shown that it results in a synchronous wave of apoptosis in the ORN population in the OE ipsilateral to the lesion, followed by near-complete regeneration of the OE over a stereotyped time course (Costanzo and Graziadei 1983; Schwartz Levey et al. 1991; Carr and Farbman 1992; Schwob et al. 1992; Holcomb et al. 1995; Leung et al. 2007; Iwai et al. 2008).

The details of neuronal regeneration following bulbectomy involve the proliferation and differentiation of a defined sequence of cellular intermediates, most of which appear to be the same cell types that have been identified in tissue-culture studies of OE neurogenesis and during OE development. Induction of ORN death, which peaks at about two days post-bulbectomy (Holcomb et al. 1995), leads to increased replicative and differentiative divisions of *Mash1*-expressing progenitors and their progeny, the INPs (schematized in Figure 10.1C; cf. Schwartz Levey et al. 1991; Gordon et al. 1995; Holcomb et al. 1995). As new ORNs are generated, the rate of progenitor cell divisions decreases until steady state is restored, about ten days after surgery in bulbectomized mice (Schwob et al. 1992; Holcomb et al. 1995; Calof et al. 1996a). These observations suggest that progenitor cells in the OE are able to "count" the number of ORNs present in the epithelium, and respond by altering their rates of division and differentiation appropriately. Indeed, tissue-culture studies have shown that purified OE neuronal progenitor cells, whose ability to generate ORNs can be quantified in neuronal "colony-forming assays," show reduced levels of neurogenesis when grown in the presence of large numbers of ORNS, indicating that ORNs produce a signal(s) that inhibits neurogenesis by their own progenitors (Figure 10.2A; Mumm et al. 1996).

This ability to "sense" changes in ORN number is presumably essential for the OE's ability to respond to fluctuations of neuronal number that occur as the OE is subjected to infection and toxic insults during the normal course of life (Hinds et al. 1984; Mackay-Sim and Kittel 1991). As described below, the response to changes in ORN number appears to be mediated by a network of signaling molecules that are expressed by, and act upon, cells within the OE itself. Recent studies using mouse genetics and tissue-culture approaches, as well as computational modeling, have begun to shed light on how the integrated action of these endogenous signaling molecules, as well as their interaction with transcriptional effectors such as *Foxg1*, coordinate replicative and differentiative divisions of OE stem and progenitor cells to control both the sizes of different OE neuronal cell populations and the morphogenesis of the olfactory mucosa and nasal cavity.



**FIGURE 10.2** Regulators of OE neurogenesis in vitro. (A) Addition of a neuronal cell fraction containing >75% ORNs to cultures of isolated OE stem/progenitor cells inhibits neurogenesis (quantified as neuronal colony-forming units) by the stem/progenitor cells. (B) Signaling proteins that promote prolonged proliferation of INPs in vitro. Only FGFs were found to have statistically significant effects on INP proliferation. (Adapted from [A] Mumm, J.S., Shou, J., and Calof, A.L., *Proc. Natl. Acad. Sci. USA*, 93, 11167–72, 1996; [B] DeHamer, M.K. et al. *Neuron*, 13, 1083–97, 1994.)

## 10.2 ENDOGENOUSLY EXPRESSED SIGNALING MOLECULES REGULATE ONSET AND MAINTENANCE OF NEUROGENESIS

#### 10.2.1 MESENCHYME-DERIVED FACTORS SUSTAIN PROLONGED NEUROGENESIS IN VITRO

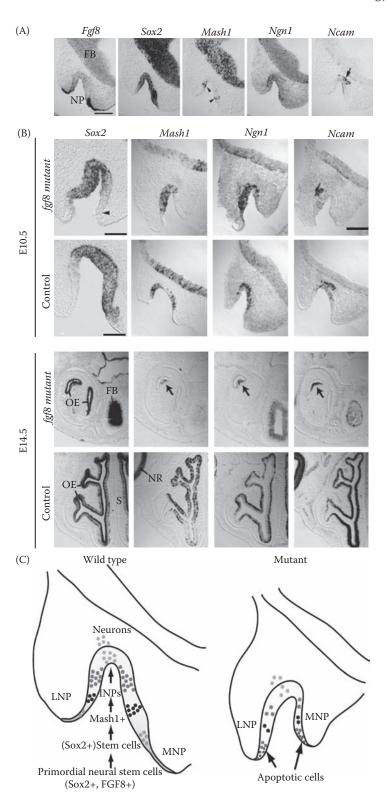
Although the OE is able to sustain de novo neurogenesis throughout life, cultured OE rapidly loses its ability to produce neurons. In serum-free medium, cultured OE stem and progenitor cells undergo differentiative divisions rather than replicative divisions, leading to depletion of stem and progenitor cells (Calof and Chikaraishi 1989). To identify conditions that would lead to sustained stem/progenitor cell activity in culture, Mumm et al. (1996) developed methods to purify (>96%) OE stem and progenitor cells by immunological "panning," depleting dissociated OE cells of ORNs using anti-NCAM antibodies immobilized on petri dishes. When these cells were cultured on top of a feeder layer consisting of cells from the olfactory stroma (mesenchyme-derived cells that underlie that OE proper), stem and progenitor cells were able to sustain proliferation and ORN production for as long as two weeks in culture (Mumm et al. 1996; Shou et al. unpublished observations). The results from this study led to the hypothesis that at least some of the factors that promote stem cell maintenance and the OE's capacity for regeneration are produced in the underlying mesenchyme (and, during postnatal life, the lamina propria of the olfactory mucosa).

## 10.2.2 FGF8 is a Positive Autocrine Regulator of Primary Olfactory Neurogenesis in Vivo

Experiments using primary OE cultures have shown that several members of the fibroblast growth factor (FGF) signaling family promote proliferation of OE stem and progenitor cells (Figure 10.2B; DeHamer et al. 1994). FGFs comprise a large family of secreted signaling proteins that have been implicated in controlling cell replication, differentiation, and survival in almost all tissues (Ornitz 2000). In OE cultures, FGFs were found to promote sustained proliferation of both stem cells and INPs. Detailed examinations demonstrated that FGFs act on INPs by preventing cell cycle exit, thereby increasing the likelihood that INPs will undergo a second round of replicative division before undergoing terminal differentiation into NCAM-expressing ORNs (DeHamer et al. 1994).

Which FGF is responsible for stem and progenitor cell maintenance in vivo? A number of Fgfs are expressed in and around the OE in vivo, during development and in postnatal life (DeHamer et al. 1994; LaMantia et al. 2000; Bachler and Neubuser 2001; Hsu et al. 2001; Kawauchi et al. 2005). Molecular analyses have revealed that Fgf8 is highly expressed at early stages of OE development, during primary neurogenesis; and that it is expressed in Sox2+ primordial neural stem cells in the epithelial margins of the invaginating olfactory pit (Figure 10.3A; Bachler and Neubuser 2001; Kawauchi et al. 2005). Other Fgfs are expressed in the OE at later times in development. For example, Fgf18 is expressed in the OE during the final third of embryonic development (Kawauchi et al. 2005); and Fgf2 expression within OE proper cannot be detected until postnatal life (Hsu et al. 2001; Kawauchi et al. 2004). Thus, it seems likely that expression of different FGFs occurs during different time periods, and/or in different cell types, during development, and regeneration of the OE. Moreover, these observations imply that different FGFs play different roles in controlling the proliferation, differentiation, and/or survival of different OE cell types.

Partial data exist on which FGFs are required for OE development and neurogenesis. Genetic experiments have shown that Fgf8 is crucial for both OE neurogenesis and nasal cavity morphogenesis during embryonic development. Importantly, the role of FGF8 in these processes is not that of a mitogen. Rather, expression of Fgf8 is required for the survival of Sox2-expressing primordial neural stem cells of the OE (Figure 10.3C; Kawauchi et al. 2005). These Sox2+ stem cells form the foundation of the OE neuronal lineage, and in the absence of Fgf8, they undergo apoptosis. The result of this event, which occurs during invagination of the olfactory pit at days 10–12 of gestation, is cessation of both OE neurogenesis and morphogenesis of the nasal cavity and olfactory mucosa.



Mice with deletion of Fgf8 in anterior neural regions survive to birth, but have virtually no nasal cavity and no OE (Figure 10.3; Kawauchi et al. 2005). Thus, since Fgf8 is expressed in the very cells (*Sox2*-expressing primordial neural stem cells) whose survival it maintains, we think of FGF8 as a positive autocrine regulator of neurogenesis that acts during the initial establishment of the OE neuronal lineage.

## **10.3 REGULATION OF NEUROGENESIS BY NEGATIVE FEEDBACK**

## 10.3.1 NEURONAL CELL-DERIVED FACTORS INHIBIT PROGENITOR CELL PROLIFERATION IN VITRO

Signals that mediate negative feedback of neurogenesis are as important in OE development and regeneration as those that promote neurogenesis. Although the temporal and spatial relationship between induced apoptosis of ORNs and proliferation of progenitor cells/ORN regeneration (Figure 10.1) suggest that those cells of the OE that are more differentiated (i.e., INPs and ORNs) feed back to inhibit proliferation and neuron genesis by proliferating progenitor cells, this concept was not tested directly until about 13 years ago. Mumm and colleagues performed experiments in which they showed that adding large numbers of neuronal cells (comprised of approximately 75% ORNs) to cultures of isolated OE neuronal progenitor cells suppresses neurogenesis by the isolated progenitors (Figure 10.2A; Mumm et al. 1996). Additional biochemical tests indicated that the neuronal cell-derived signal was a polypeptide (Calof et al. 1996b), and led to the examination of transforming growth factor (TGF- $\beta$ ) superfamily signaling molecules as candidates for the antineurogenic feedback factor(s) of the OE.

## 10.3.2 Autoregulation of Neurogenesis by GDF11

Studies using primary OE cultures have identified several members of the TGF- $\beta$  family of signaling molecules as potent negative regulators of OE neurogenesis (DeHamer et al. 1994; Shou et al. 1999, 2000; Wu et al. 2003). Indeed, of the numerous signaling molecules assessed in an early screen to test for factors affecting immediate neuronal precursor (INP) proliferation (Figure 10.2B), the only factor to have a negative effect on INP proliferation was TGF- $\beta$ 1 (DeHamer et al. 1994). TGF- $\beta$ s comprise a large superfamily of secreted signaling molecules that have been implicated in regulating proliferation, differentiation, and cancer in virtually all tissues (Hogan 1996; Massague et al. 2000; Chang et al. 2002; Feng and Derynck 2005; Liu and Niswander 2005). A number of different TGF- $\beta$ s are expressed in OE proper and its underlying mesenchymal stroma, and studies

FIGURE 10.3 (Opposite) (See color insert following page 206.) Absence of Fgf8 leads to apoptosis of primordial Sox2-expressing OE neural stem cells and cessation of OE neurogenesis and nasal cavity morphogenesis. (A) Expression of Fgf8 and neuronal lineage markers in E10.5 olfactory pit (in situ hybridization, ISH). Note overlap between Fgf8 and Sox2 expression domains. Arrowheads: Mash1-expressing cells; arrow: Ncam-expressing neurons. FB, (presumptive) forebrain; NP, nasal pit. Scale bar: 200 µm. (B) Cessation of neurogenesis in Fgf8 mutants (conditional allele of Fgf8 deleted using BF1-Cre) (Hebert and McConnell 2000). Arrowhead marks reduced Sox2 expression in the OE lining the nasal pit at E10.5; arrows indicate apparent OE remnant in E14.5 mutant animals. FB, forebrain; NP, nasal pit; NR, neural retina; OE, olfactory epithelium; S, nasal septum. Scale bars: 200 µm. (C) Schematic of FGF8's role in OE neurogenesis. The sketch shows the relative positions of different neuronal cell types within the OE during primary olfactory neurogenesis at E10.5 in wildtype and Fgf8 mutants. Fgf8 expression domain, orange; Sox2 expression domain (definitive neuroepithelium), yellow; Sox2+ stem cells, green; Mash1+ early progenitors, dark blue; Ngn1+ INPs, light blue; Ncam+ ORNs, pink. Cells in the Fgf8-expressing domain that undergo apoptosis when Fgf8 is inactivated are shown in red, and apoptotic primordial neural stem cells (Sox2+, Fgf8+) are in green with red jagged border. Vestigial populations of other neuronal cell types are shown in their corresponding colors, but with jagged borders. ([C] Adapted from Kawauchi, S. et al. Development, 132, 5211–23, 2005.)

have shown that these have diverse effects on OE neurogenesis, including control of development of *Mash1*-expressing progenitors and effects on ORN survival (Shou et al. 1999, 2000; Wu et al. 2003; Kawauchi et al. 2004).

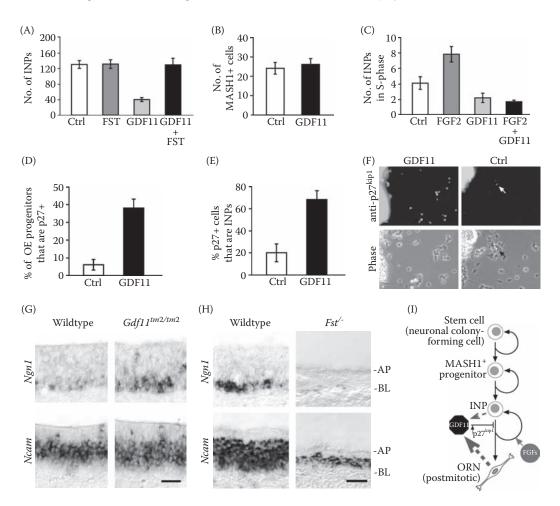
Using a candidate approach to identify the signal(s) responsible for endogenous negative feedback of neurogenesis in the OE, Wu and colleagues focused on growth and differentiation factor 11 (GDF11), a member of the activin-like family of TGF- $\beta$ s, which signal intracellularly via Smads2 and 3 (Andersson et al. 2006; Massague and Gomis 2006). A primary reason for focusing on GDF11 was its extensive homology to GDF8 (myostatin), an autocrine negative regulator of skeletal muscle cell growth (Lee and McPherron 1999; Nakashima et al. 1999; Wu et al. 2003). During development, Gdf11—which is expressed specifically in the OE neuroepithelium proper, and there primarily by immature ORNs and neuronal progenitors-plays a crucial role in the negative regulation of neuron number (Nakashima et al. 1999; Gamer et al. 2003; Wu et al. 2003). In vitro, GDF11 induces complete, but reversible, inhibition of INP cell divisions, without affecting development of their precursors, the Mash1-expressing early progenitor cells (Figure 10.4A and B; Wu et al. 2003). Interestingly, GDF11's antiproliferative effect is able to override the positive effect of FGFs on INP proliferation, which have been described previously (see above and DeHamer et al. 1994), indicating that INPs must integrate signals from the FGF and TGF- $\beta$  different signaling pathways to control their proliferation and growth. INP cell cycle arrest appears to be mediated by GDF11induced upregulation of the cyclin-dependent kinase inhibitor, p27<sup>Kipl</sup>, which arrests cells in G1 phase (Figure 10.4D through F; Chen and Segil 1999; Levine et al. 2000; Miyazawa et al. 2000; Dyer and Cepko 2001).

Importantly, the negative regulation of neurogenesis affected by GDF11 is also observed in vivo. In mice that are made null for Gdf11 ( $Gdf11^{im2/tm2}$  mice), the second reported null allele of Gdf11 (Wu et al. 2003), the OE contains increased numbers of INPs and ORNs and shows an increase in overall thickness compared to wildtype littermates (Figure 10.4G). Just as has been shown in vitro, however, there appears to be no change in the number of *Mash1*-expressing cells in the OE of Gdf11-null mice (Wu et al. 2003). Thus, GDF11 acts as an autocrine negative regulator of neuron number during OE development, and its actions appear to be directed at INPs, the immediate precursors of ORNs. A schematic showing the present concept of how GDF11 acts in regulating feedback inhibition of neurogenesis is shown in Figure 10.4I.

*Gdf11* expression in the OE can be detected as early as day 10.5 of development (Kawauchi et al. 2009), and it continues to be expressed through development and adulthood. How is neurogenesis able to proceed in a tissue that expresses persistent levels of antineurogenic factors such as GDF11? Since *Gdf11* transcripts can be detected in a number of neural regions, in addition to OE, in which robust neurogenesis occurs even into postnatal periods (Nakashima et al. 1999; Kim et al. 2005; Wu and Calof unpublished observations), GDF11 activity must be tightly regulated in order for appropriate progenitor cell proliferation to be maintained and proper neuron number achieved.

## 10.3.3 FOLLISTATIN (FST), A GDF11 ANTAGONIST, PROVIDES A PERMISSIVE ENVIRONMENT FOR NEUROGENESIS

Follistatin (FST), a secreted protein, has been shown to antagonize signaling by a number of different activin-like TGF- $\beta$ s, including activins themselves, GDF8, GDF11, and BMP7 (Gamer and Rosenblatt 1986; Schneyer et al. 1994, 2008; Gamer et al. 1999; Lee and McPherron 2001; Rebbapragada et al. 2003; Wu et al. 2003). *Fst*, which is expressed in OE and its underlying stroma, antagonizes activin-like TGF- $\beta$ s by binding to them and preventing signaling through their receptors (Sugino et al. 1997; Phillips and de Kretser 1998; Schneyer et al. 2003). In OE cultures, the addition of FST abrogates GDF11's antiproliferative effects on INPs (Figure 10.4A), suggesting that FST's presence in vivo may be of importance for controlling the magnitude and extent of GDF11 antineurogenic signaling. Indeed, when the OE of mice null for *Fst* (*Fst*<sup>-/-</sup> mice;



**FIGURE 10.4** Roles of GDF11 and FST in regulating INP development and OE neurogenesis. (A) Development of INPs in culture is inhibited in the presence of GDF11. This effect is abrogated by the addition of FST. (B) GDF11 does not affect the development of MASH1+ progenitor cells. (C) GDF11 prevents FGF2-stimulated proliferation of INPs. (D, E) Many OE neuronal progenitor cells are induced to express  $p27^{Kip1}$  in GDF11-treated OE cultures; most of these are INPs. (F) Addition of GDF11 induces  $p27^{Kip1}$  expression in INPs in OE cultures. (G) *Gdf11<sup>im2/</sup>* <sup>1m2</sup> (*Gdf11* null exhibit increased OE neurogenesis, as shown by the increase in the numbers of *Ngn1-* and *Ncam*-expressing cells in the OE. (H) Mice null for *Fst* show decreased neurogenesis; GDF11, which is produced by INPs and ORNs, inhibits division of INPs via upregulation of  $p27^{Kip1}$ . Conversely, FGFs promote INP proliferation. (Adapted from Wu, H.H. et al. *Neuron*, 37, 197–207, 2003.)

Matzuk et al. 1995) was examined, it was found to have severely decreased numbers of INPs and ORNs, as well as a much thinner OE than that observed in wildtype littermates (Figure 10.4H; Wu et al. 2003). Thus, current thinking suggests that the presence of FST within the nasal mucosa is crucial for maintaining an environment permissive for OE neurogenesis (see also Figure 10.9A; Kawauchi et al. 2009).

Recent evidence indicates that GDF11 is not the only antineurogenic factor that is regulated by FST in the OE. In addition to INPs, both *Sox2-* and *Mash1*-expressing stem/early progenitor cells are also depleted dramatically in *Fst<sup>-/-</sup>* OE (Wu and Calof, unpublished observations). As all available data indicate that the antineurogenic effects of GDF11 are limited to cells downstream of *Mash1*-expressing progenitors in the ORN lineage, these observations suggest that another molecule, whose signaling is antagonized by FST, controls the divisions of *Sox2*- and *Mash1*-expressing early stem and progenitor cells. Accordingly, recent experiments indicate that activins themselves are expressed within the nasal mucosa, and have negative effects on stem/progenitor cell proliferation in OE cultures (Gokoffski and Calof, unpublished observations).

Although the observations described above come from studies of developing OE, Gdf11 and Fst continue to be expressed in the adult (Gokoffski and Calof, unpublished observations), suggesting that they play a significant role in mediating the controlled and coordinated regeneration that is observed in injured adult OE. Testing such predictions has been limited by the fact that  $Gdf11^{-/-}$  and  $Fst^{-/-}$  mice die at birth, for reasons unrelated to their effects on OE (Matzuk et al. 1995; McPherron et al. 1999; Esquela and Lee 2003; Wu et al. 2003). Development of conditional mutant alleles that allow for tissue-specific inactivation of Gdf11 and Fst will permit experiments to be performed that should provide important insights for understanding how regeneration is controlled and how stem/progenitor activity is coordinated with ORN number during this process (Jorgez et al. 2004). Since GDF11 has also been shown to be a regulator of stem cell fate in another sensory neuroepithelium, the retina (Kim et al. 2005), it will be interesting to see if GDF11 plays such a role in OE regeneration.

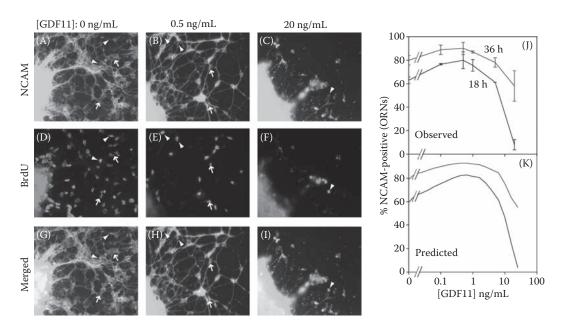
## 10.4 COMPUTATIONAL APPROACHES SUGGEST CRUCIAL ROLES FOR NEGATIVE FEEDBACK IN ACHIEVING RAPID AND ACCURATE REGENERATION IN THE OLFACTORY EPITHELIUM (OE)

## 10.4.1 GDF11 CONTROLS THE RATIO OF PROLIFERATIVE VS. DIFFERENTIATIVE DIVISIONS OF IMMEDIATE NEURONAL PRECURSOR CELLS

The location of the OE within the nasal cavity exposes it directly to the environment, making it vulnerable to random environmental assaults, which in turn leads to highly variable rates of ORN loss. Such unpredictability creates significant challenges for the homeostatic control of ORN number. Yet, the OE performs remarkably well: even when virtually all ORNs are eliminated acutely, ORN numbers are restored rapidly and without substantial overshoot (Schwartz Levey et al. 1991; Carr and Farbman 1992; Holcomb et al. 1995; Ducray et al. 2002; Costanzo and Graziadei 1983; Schwob et al. 1995). Since olfaction is crucial for the survival of many animals, rapid and accurate regeneration of ORNs has obvious evolutionary advantages. Can we directly relate the feedback provided by molecules produced within the OE, such as GDF11, activins, and FST, to the rapidity and accuracy of regeneration?

The question of how dynamic processes, such as feedback, enable systems to achieve goals such as robustness, efficiency, and speed, is a major focus of systems biology, and is often approached with the help of mathematical and computational modeling. We recently applied such methods to the analysis of feedback within the OE neuronal lineage (Lander et al. 2009), with striking results. The first thing we learned was that, if the sole action of GDF11 is to regulate the rate of INP cell divisions (as had been shown in vitro; Wu et al. 2003), then GDF11 could contribute nothing to steady-state homeostasis (i.e., maintaining a desired number of ORNs despite variable environmental challenges). Moreover, its contributution to increasing overall speed of regeneration would be modest at best.

Further modeling led us to predict that GDF11 has an additional action: controlling the proportion of INP daughters that become ORNs instead of continuing to divide and becoming more INPs (cf. Figure 3 in Lander et al. 2009). When tissue-culture experiments were performed to test this hypothesis directly, they demonstrated that GDF11 does indeed control INP differentiation, in a dose-dependent manner (Figure 10.5): treatment with low concentrations of GDF11 (0.1–1 ng/mL) pushes INPs to differentiate into NCAM-expressing ORNs; whereas high doses of GDF11 (20 ng/ mL) in these same cultures prolongs INP cell-cycle length, delaying differentiation of these cells to ORNs (Figure 10.5). Significantly, these actions of GDF11 occurred over the same time course that was predicted from modeling (Figure 10.5; Lander et al. 2009). Thus, GDF11 has two major



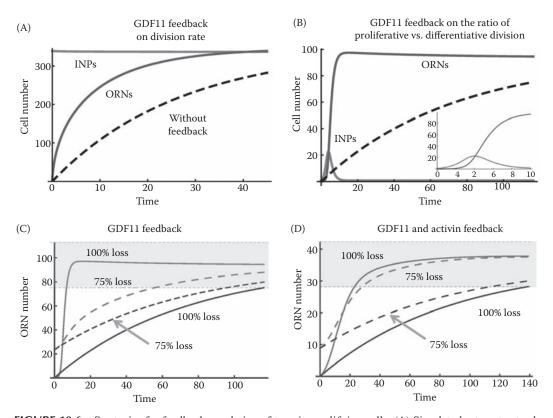
**FIGURE 10.5** (See color insert following page 206.) GDF11 regulates ratio of INP proliferative vs. differentiative divisions. (A–J) At low doses of GDF11, the proportion of INP progeny that differentiate into ORNs increases. At high doses, the effect reverses, with the NCAM<sup>+</sup> fraction falling to near zero at 18 h, but recovering at 36 h. The reversal is consistent with a slowing of the cell cycle such that 18 h is insufficient for the production of NCAM-expressing, terminally differentiated ORNs (but 36 h is). This interpretation is consistent with previous data demonstrating that high doses of GDF11 reversibly arrest the INP cell cycle (Wu et al. 2003). (K) Simulation of the experiment in (J) by a model in which GDF11 affects both ratio of proliferative vs. differentiative divisions and division rate. ([K] Reprinted from Lander, A.D. et al. *PLoS Biol.*, 7, e15, 2009.)

functions in feedback control of neurogenesis: to control the ratio of replicative vs. differentiative divisions of INPs, and to control the cell-cycle length of INPs.

Modeling shows that the effects of these two mechanisms on regeneration (modeled as an acute loss of most ORNs) will be profoundly different. Whereas feedback on INP division rate exerts no control over steady-state ORN numbers, feedback on the replication/differentiation choice of INPs can lead to nearly perfect control (maintaining correct ORN number independent of fluctuations in rates of ORN death, or even in numbers of stem cells or rates of stem cell division). Likewise, the modest improvement in regeneration speed that is provided by feedback on INP division rate comes at the expense of a requirement that a very large fraction of the tissue needs to consist of INPs. As shown in Figure 10.6A, in order to drive regeneration that is threefold faster than the normal rate of ORN turnover, half the OE would need to be INPs (in reality, that number is probably less than 10%) (Smart 1971; Cuschieri and Bannister 1975; Mackay-Sim and Kittel 1991; Schwartz Levey et al. 1991; Farbman 1992; Gordon et al. 1995; Mumm et al. 1996). In contrast, with feedback on the replication/ differentiation choice of INPs, regeneration can occur up to 100% times faster than the normal rate of ORN turnover, and only a small fraction of the cells in the tissue need to be INPs (Figure 10.6B; cf. Lander et al. 2009). Moreover, regeneration under such circumstances will be characterized by a transient expansion and then contraction of the INP pool, followed by a large increase in ORN number; this is just the sort of behavior the OE displays following bulbectomy (Figure 10.1).

#### 10.4.2 MULTIPLE FEEDBACK LOOPS IMPROVE PERFORMANCE

Although modeling demonstrated that GDF11, by virtue of its ability to regulate the choice between replication and differentiation by INPs, could achieve important goals of speed and robustness in the



**FIGURE 10.6** Strategies for feedback regulation of transit-amplifying cells. (A) Simulated return to steady state, after removal of all ORNs, of a system with negative feedback regulation on the INP cell-cycle length (i.e., division rate). Note that feedback leads to modestly improved regeneration speed (over what would occur in the absence of feedback; dashed line), but only when INP numbers are almost as high as those of ORNs. (B) Simulated return to steady state, after removal of all ORNs, of a system with negative feedback regulation of the ratio of INP proliferative vs. differentiative divisions. Note the much greater improvement in regeneration speed (over absence of feedback; dashed line) without necessitating a high INP reserve. Inset shows response at early times in greater detail. (C) Dependence of rate of regeneration on the severity of initial ORN depletion, for the case shown in (B). Notice how the rate of return to steady state after a partial (75%) ORN loss (dashed gray curve) is only slightly better than in the absence of feedback (dashed black curve). (D) Simulated regeneration experiment similar to that in (C), except that both GDF11 and activin feedback loops are included in the model. Now, regeneration following 75% ORN depletion is almost as fast as from 100% depletion (compare with [C]). (Adapted from Lander, A.D. et al. *PLoS Biol.*, 7, e15, 2009.)

OE, further analysis revealed several problems: First, it was not possible to find conditions (numbers of cells, strengths of feedback, etc.) under which both speed and robustness could be achieved at the same time. Second, we learned that the ability to achieve explosively fast regeneration following a total loss of ORNs, only came at the expense of condemning the system to very slow regeneration following a less-than-total loss of ORNs (e.g., a 75% reduction; Figure 10.6C).

Interestingly, both of these obstacles can be overcome by introducing a second feedback loop into the system—this time directed at the behavior of the *Sox2-* and *Mash1*-expressing cells that are the progenitors of INPs (Figure 10.6D). Such cells are not responsive to GDF11, but, as mentioned earlier, respond to activins, which are also produced in the OE (Gokoffski and Calof, unpublished observations). Altogether, these findings indicate that multiple feedback loops are necessary to make the OE robust to a large range of environmental perturbations, and to permit the rapid and controlled regeneration of ORNs, which is such an important characteristic of this sensory neuroepithelium.

## **10.4.3** FOLLISTATIN (FST) EXPRESSION CREATES A STEM CELL NICHE IN THE OLFACTORY EPITHELIUM (OE)

If the purpose of feedback is to report to stem and progenitor cells the overall tissue size and/or number of ORNs, then the concentrations of feedback molecules that are sensed by stem and progenitor cells need to vary proportionally (or nearly so) with tissue size and/or ORN number. Within a tissue, a secreted molecule's local concentration depends on its rate of production, diffusivity, and rate of uptake and degradation, but also on what happens to it when it reaches the boundaries of the tissue. If those boundaries are closed (i.e., the molecule cannot escape), then no matter how the tissue may grow in size, the concentration of any molecule secreted uniformly throughout the tissue will remain unchanged (this is because the volume in which the molecule is diluted goes up at the same rate as the amount of the molecule that is produced). In such a tissue, levels of secreted molecules can never provide feedback information about tissue size or numbers of terminally differentiated cells.

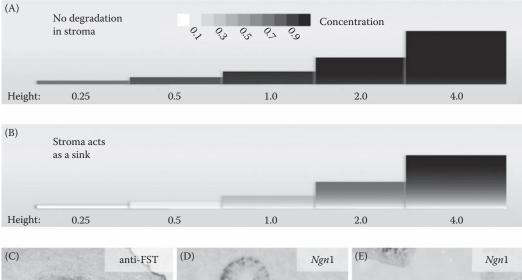
Few tissues are truly "closed," but epithelia are effectively closed at one end (the apical end, where tight junctions exist), and open at the other (the basal lamina), which is freely permeable to polypeptide growth factors (Dowd et al. 1999). We can calculate how the levels of secreted molecules will vary with tissue size (epithelial thickness) for such an arrangement, but only after first specifying what happens to signaling molecules after they cross the basal lamina. If we assume that they are free to wander back across into the epithelium, we get the result shown in Figure 10.7A, in which the concentration of the growth factor within the epithelium starts to plateau when the epithelium is rather small. In effect, even though the epithelium is open at one end, it behaves as if closed once it has grown beyond a certain thickness (this thickness corresponds to about half the mean distance the signaling molecule travels within the epithelium before it is captured by receptors; this is a distance that can be estimated to be in the tens of micrometers; Lander et al. 2009). In contrast, if we specify that the growth factor is quickly and irreversibly trapped (or degraded) once it crosses the basal lamina, we get the results shown in Figure 10.7B. Now, growth factor concentration within the tissue rises over a much larger range of epithelial sizes, allowing such a growth factor to be a useful reporter of tissue size.

Such calculations are instructive because they provide a logical explanation for the localization of FST expression in the OE. As shown in Figure 10.7C, the major source of FST associated with the developing OE is in the stroma beneath the epithelium (even though genetic experiments, discussed above, show that it acts on GDF11 and activins produced within the epithelium). Since FST is known to be an irreversible binder of activins and GDF11 (Schneyer et al. 2008), it can be expected to provide just the sort of sink portrayed in Figure 10.7B, allowing such molecules to be efficient reporters of OE size (and ORN numbers).

This sort of analysis illustrates how genetics and modeling can give different, yet complementary, views of the same process. From the standpoint of genetics, FST is an inhibitor of GDF11 and activins in the OE. Modeling, however, suggests that the primary role of FST may be less to inhibit these molecules than to alter their distribution within the OE. An important consequence of this effect is to create a defined region—just above the basal lamina—where the effective concentrations of GDF11 and activins are lowest, and also vary most sensitively with epithelial size (Lander et al. 2009). Remarkably, this is precisely where stem and progenitor cells (the cells that respond to GDF11 and activins) come to reside in the OE (Figures 10.7D and E). Through its action in the stroma, FST effectively creates a stem/progenitor cell "niche" within the epithelium, where such cells are most able to proliferate, and most efficient in responding to perturbations in OE size or ORN number.

## 10.4.4 CONSEQUENCES OF FEEDBACK FOR UNDERSTANDING STEM VS. TRANSIT-AMPLIFYING CELLS

Recent evidence suggests that differing levels of expression of *Sox2* and *Mash1* may actually represent alternative states of a single stem/early progenitor cell, whereas *Ngn1*-expressing INPs



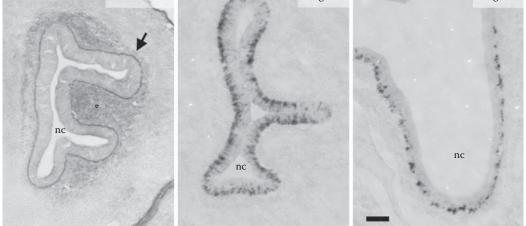


FIGURE 10.7 Effects of geometry and degradation on levels of secreted molecules within epithelia. (A, B) Two processes remove polypeptides secreted into the intercellular space of an epithelium: diffusion into underlying connective tissue (stroma) and degradation within the epithelium. Given a molecule's rate of production, its diffusivity, its rate of uptake and degradation, and the geometry of the epithelium, one may calculate its steady-state distribution. Here, such calculations are shown graphically, for epithelia of different thicknesses (in each picture the epithelium is oriented with the apical surface at the top). Epithelial thickness ("height") is scaled according to the decay length of the molecule of interest. The shading in each picture depicts the concentration of the secreted molecule, with black representing the limiting concentration that would be achieved in an epithelium of infinite thickness. (A) The degradation capacity of the stroma is set at one-tenth that in the epithelium. In this case, intraepithelial concentrations of secreted molecules plateau while the epithelium is very thin. (B) The stroma is treated as a strong sink, i.e., few molecules that enter it escape undegraded. Now there is a large (and more physiological) range of epithelial thickness over which the concentrations of secreted molecules grow appreciably with tissue size. This is particularly true near the basal surface of the epithelium. (C) Follistatin (FST), a molecule that binds GDF11 and activin essentially irreversibly, is present at high levels in the basal lamina (arrow) and stroma (asterisk) at E13 OE. Size bar: 100 µm. (D, E) INPs (visualized with Ngn1 in situ hybridization) become progressively localized to the basal surface of the OE over the course of development. (D)=E12.5; (E)=E18.5. nc=nasal cavity. Size bar: 100 µm. (Adapted from Lander, A.D. et al. PLoS Biol., 7, e15, 2009.)

are clearly a separate population with distinct properties and roles in development and regeneration (Murray et al. 2003; Wu et al. 2003; Beites et al. 2005; Gokoffski and Calof, unpublished observations). The fact that *Sox2/Mash1*-expressing cells give rise to INPs (which exit the cell cycle to differentiate into ORNs) might tempt classification of these as stem cells (cells that divide indefinitely and asymmetrically), and Ngn1-expressing cells as transit-amplifying cells (cells that are committed to a single differentiative endpoint and can only undergo limited rounds of division) (DeHamer et al. 1994; Gordon et al. 1995). However, modeling of cell lineages suggests that such behaviors are not likely to be intrinsic properties inherent to each cell population, but rather, may be characteristic behaviors of cells that occur as a consequence of feedback regulation (Lander et al. 2009). Such models of the ORN lineage and other lineages show that if stem and progenitor cells self-replicate more than half the time, then negative feedback modulation of their behaviors is sufficient to give rise to a system in which the first cell stage (Sox2/Mash1-expressing cells) self-replicates exactly half the time; while the second cell stage (Ngn1-expressing cells) undergoes an apparently limited number of cell divisions (Shen et al. 2006; Lander et al. 2009). Moreover, such modeling predicts that the "stem" cell stage can extinguish itself in such systems, which will cause the second cell stage (the "transit-amplifying cell") to adopt "stem-like" behavior. Thus, the behaviors that we think of as characterizing stem vs. transit-amplifying cell populations in regenerating tissues (Potten 1981; Jones and Watt 1993) may not be immutable, intrinsic characteristics of the cells, but rather the outcomes of these cells' responses to extrinsic signals, such as GDF11 and activin. Ultimately, these studies suggest that using cell cycle characteristics to define stem vs. transit-amplifying cells may not be the most useful means of understanding the regenerative properties of tissues (Lander et al. 2009).

## 10.5 OTHER TYPES OF FEEDBACK: INTERACTION OF TRANSFORMING GROWTH FACTOR (TGF- $\beta$ )s WITH NEURAL SPECIFICATION FACTORS

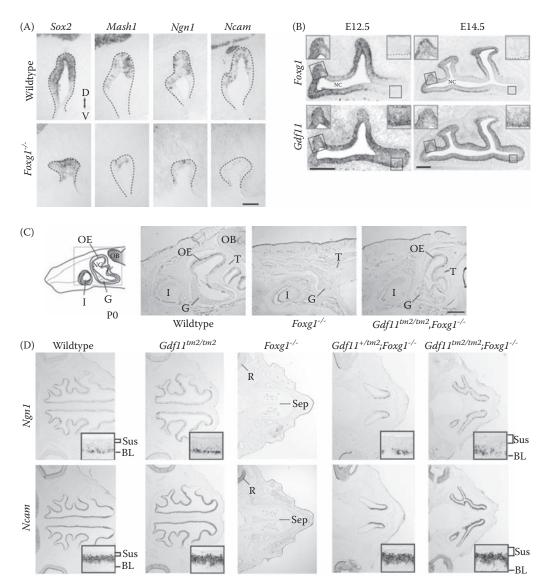
## 10.5.1 OLFACTORY EPITHELIUM (OE) FORMATION REQUIRES FOXG1

*Foxg1* (Forkhead box factor G1, also known as Brain Factor-1; Carlsson and Mahlapuu 2002) is a homeobox transcription factor that is highly expressed in embryonic forebrain and has been reported to promote development of several anterior neural structures (Xuan et al. 1995; Hebert and McConnell 2000; Hanashima et al. 2004, 2007; Pratt et al. 2004; Martynoga et al. 2005; Pauley et al. 2006; Duggan et al. 2008). Mice null for *Foxg1* show dramatic reductions in the size of their cerebral hemispheres and are missing ventral telencephalic structures, and die shortly after birth (Xuan et al. 1995). The OE of *Foxg1-/-* animals is also greatly diminished or even absent, due to defects that occur early in development. In *Foxg1-/-* OE, cells expressing ORN lineage markers are present, but already greatly reduced in number by day 11 of gestation. As shown in Figure 10.8A, only a few *Mash1*-expressing early progenitors can be detected in a restricted domain in *Foxg1-/-*OE at E11, and *Ngn1-* and *Ncam*-expressing INPs and ORNs are even more dramatically reduced (Kawauchi et al. 2009). OE neurogenesis and nasal cavity morphogenesis both cease early in development in *Foxg1-/-* mice, such that, by E13.5, *Foxg1-/-* mice lack an OE and most of their nasal cavity (Xuan et al. 1995; Duggan et al. 2008; Kawauchi et al. 2009).

## 10.5.2 INTERACTION OF *GDF11* AND *FOXG1* REGULATES HISTOGENESIS AND MORPHOGENESIS OF THE OLFACTORY EPITHELIUM (OE) AND NASAL CAVITY

Experiments using cultured neuroepithelial cells have demonstrated that FoxG1 can bind to Smad3containing complexes (Smad3 has been established as a component of the GDF11 cytoplasmic signaling pathway; Oh et al. 2002; Andersson et al. 2006) and thus block expression of *p21Cip1*, which encodes a cyclin-dependent kinase inhibitor that is known to be an effector of both GDF11 and TGF- $\beta$  signaling (Nomura et al. 2008; Tsuchida et al. 2008). Since *p21Cip1* is also known to be expressed in the OE (Kastner et al. 2000; Legrier et al. 2001), Kawauchi and colleagues hypothesized that interactions of FoxG1 with GDF11 might be important in the regulation of OE development by FoxG1. Analysis of *Foxg1* expression in the OE using *in situ* hybridization revealed that the expression domain of *Foxg1* overlaps substantially with that of *Gdf11* throughout much of prenatal OE development (Figure 10.8B; Kawauchi et al. 2009). Transcripts of both *Gdf11* and *Foxg1* are predominantly restricted to the basal compartment of the epithelium, where stem and neuronal progenitor cells are located. However, the expression of *Foxg1* within the OE is not uniform: by E12.5, there are clear regional differences, with *Foxg1* expressed at greatest levels in the OE located in the recesses of the developing turbinates and the posterior recess of the nasal cavity (at the junction of the septum and turbinates; Figure 10.8B). These are the very regions of the OE that are most actively expanding into the nasal mesenchyme, as morphogenesis of the nasal cavity proceeds during prenatal development. In contrast, *Gdf11* expression is rather uniformly expressed within the OE, wherever OE is present in the nasal cavity (Figure 10.8B).

The presence of Gdf11 and Foxg1 transcripts at similar times and in the same cell populations; the known ability of FoxG1 to inhibit expression of at least some GDF11 target genes; and the opposite OE phenotypes that result from absence of Gdf11 vs. absence of Foxg1; together raise the possibility that FoxG1 regulates OE development by regulating the action of GDF11. To assess this directly, genetic epistasis experiments were performed, and the OE was analyzed in wildtype,



 $Foxg1^{-/-}$ ,  $Gdf11^{+/-}$ , and  $Foxg1^{-/-}$ ;  $Gdf11^{-/-}$  double mutants (Figure 10.8C and D). These experiments demonstrated that loss of Gdf11 rescues defects in OE neurogenesis that result from inactivation of Foxg1, and in a Gdf11 gene dosage-dependent manner. Figure 10.8C shows what this looks like at birth (P0): in wildtype OE, the olfactory turbinates are well-developed and the OE is easily recognized by in situ hybridization to the INP marker, Ngn1. In contrast, in the  $Foxg1^{-/-}$  mice, essentially no OE is present, and there are no olfactory turbinate structures in what appears to be the vestige of the nasal cavity. However, in Foxg1;Gdf11 double nulls, there is a significant rescue of both nasal cavity formation and OE development, and Ngn1-expressing cells can be observed in the basal compartment of a well-developed OE, which covers an identifiable—albeit smaller than normal—nasal cavity (Kawauchi et al. 2009).

The OE of single and double mutants, as well as *Foxg1* nulls in which only one allele of *Gdf11* was inactivated, were also examined in these studies. As shown in Figure 10.8C, cells of the ORN lineage can be easily recognized at E16.5 in wildtype animals, by their laminar positions and expression of the neuronal cell markers, *Ngn1* and *Ncam*. In contrast, OE, nasal cavity, and ORN lineage markers are all absent in *Foxg1-/-* mice at E16.5. Strikingly, when *Foxg1-/-* embryos are also made null for *Gdf11*, both the OE and the nasal cavity itself are rescued significantly. The OE of *Foxg1-/-;Gdf11-/-* mice is of normal thickness, and contains cells expressing major lineage markers (Figure 10.8D). Notably, when just one allele of *Gdf11* is inactivated in *Foxg1 null* mutants (*Foxg1-/-;Gdf11+/-* mice), both OE histogenesis and nasal cavity formation are significantly restored. The degree of rescue is more pronounced in double mutants compared to *Foxg1-/-;Gdf11+/-* compound mutants, suggesting that *Foxg1-/-* phenotypic rescue is dependent on *Gdf11* gene dosage. In addition, the fact that removal of a single *Gdf11* allele transforms the *Foxg1-/-* phenotype from one in which no nasal cavity develops, into one with a nasal cavity lined by an OE of normal thickness and composition, suggests that there is a threshold level of GDF11 activity below which histogenesis and morphogenesis can proceed fairly normally, and above which these processes fail completely.

FIGURE 10.8 (Opposite) Absence of Gdf11 rescues deficits in neurogenesis and morphogenesis observed in Foxg1-/- OE. (A) Failure of neurogenesis in Foxg1-/- OE. Sections of OE from wildtype and Foxg1-/- embryos at E11 show that olfactory pits are greatly reduced in size. The total area of Sox2-expressing neuroepithelium is also reduced in the mutant compared to wildtype. Only a few Mash1<sup>+</sup> early progenitors can be detected, and the decrease in Ngn1-expressing INPs and Ncam-expressing ORNs is even more dramatic. D, dorsal; V, ventral. Scale bar: 100  $\mu$ m. (B) Expression of *Foxg1* and *Gdf11* in developing mouse OE. Horizontal sections show the OE in one-half of the nasal region (septum is at bottom) at E12.5 and E14.5 in wildtype mice (anterior is right, posterior is left). Expression of *Foxg1* and *Gdf11* overlap except in anterior OE, which has ceased planar expansion at these ages. Insets show high magnification of the OE at posterior regions of coexpression and anterior regions where coexpression has ceased. Dotted line indicates basal lamina. NC, nasal cavity; scale bars: 200  $\mu$ m. (C) Rescue of Foxg1-/- OE phenotype by loss of Gdf11. The sketch is of a midsagittal section through the frontonasal structure of wildtype P0 mice. Box indicates region of images on left. Images show Ngn1 expression in the OE neuroepithelium where it contains neuronal progenitor cells. Olfactory turbinate structures and Ngn1-expressing INPs are not observed in Foxg1 mutants; mice that are null for Gdf11 as well as Foxg1 (Gdf11<sup>m2/tm2</sup>;Foxg1<sup>-/-</sup> mice) show recovery of turbinate structures and OE. G, serous gland; I, incisor tooth; OE, olfactory epithelium; OB, olfactory bulb; NC, nasal cavity; T, turbinate bone. (D) Rescue of OE neurogenesis in Foxg1-/- is dependent on Gdf11 gene dosage. ISH for OE neuronal lineage markers (Ngn1 and Ncam) in the OE of E16.5 wildtype and mutant littermates. Insets show high magnification views of septal OE. In Gdf11<sup>tm2/tm2</sup> mice, Ngn1- and Ncam-expressing cell layers (and hence overall OE) are thicker compared to wildtype, as reported previously (Wu et al. 2003). No discernable OE structure is evident in  $Foxg1^{-/-}$  mice at the same dorsoventral level. Loss of one allele of Gdf11 ( $Gdf11^{+/tm2};Foxg1^{-/-}$ ) rescues all cell types in the OE, and the OE appears of normal thickness, although planar expansion of the OE and morphogenesis of the nasal cavity are clearly deficient in the compound mutant. Rescue is more pronounced in double nulls (Gdf11tm2/tm2;Foxg1-/-), particularly in terms of OE planar expansion and nasal cavity morphogenesis. For all panels, posterior is left, anterior is right; scale bar: 400 µm. (Adapted from Kawauchi, S. et al. Development, 2009.)

#### 10.5.3 REGULATION OF GDF11 AND FST EXPRESSION BY FOXG1

As mentioned previously, the importance of FST as an endogenous antagonist of GDF11 signaling is evident by the deficits in neurogenesis observed in  $Fst^{-/-}$  mice, in which the OE is very thin and markedly depleted of INPs and ORNs (Figure 10.4H; Wu et al. 2003). However, nasal cavity morphogenesis appears normal in  $Fst^{-/-}$  mice, and an OE is present, although it is much thinner than normal (Figure 10.9A; Kawauchi et al. 2009). Interestingly, we found that  $Foxg1^{-/-}$  embryos lack Fst expression in and around the OE from the earliest developmental stages (Figure 10.9B). This finding suggested an additional mechanism by which Foxg1 could antagonize Gdf11 activity: by promoting expression of Fst, Foxg1 would lower the effective concentration of GDF11 in the OE. Consistent with this idea, when OE development in  $Foxg1^{-/-}$  mice is rescued by removing one or more alleles of Gdf11, Fst expression is also restored in the tissue (Figure 10.9C). Altogether, these findings indicate that the OE phenotype in  $Foxg1^{-/-}$  mice arises from a combination of intracellular (cell-autonomous) and extracellular (non-cell-autonomous) regulation of GDF11 signaling. This may explain why the absence of Foxg1 leads to a more severe phenotype in the OE than that observed in  $Fst^{-/-}$  mice.

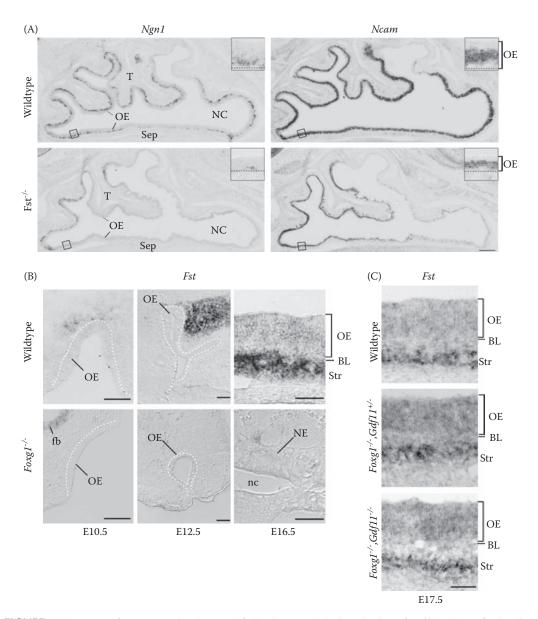
It is worthwhile noting that control of *Fst* expression by FoxG1 is unlikely to be direct. Both stromal and intraepithelial *Fst* expression are completely rescued in  $Foxg1^{-/-};Gdf11^{tm2/tm2}$  double mutants as well as in  $Foxg1^{-/-}, Gdf11^{+/-}$  compound mutants (Figure 10.9C), as mentioned above. This demonstrates that neither Foxg1 nor Gdf11 are themselves required for *Fst* expression. Rather, these findings suggest that it is the OE that is responsible for inducing and maintaining *Fst* expression in the mesenchyme, with *Foxg1* being required to generate an OE that is competent to do so.

#### 10.5.4 Foxg1 Antagonizes Gdf11 Activity Directly and Indirectly

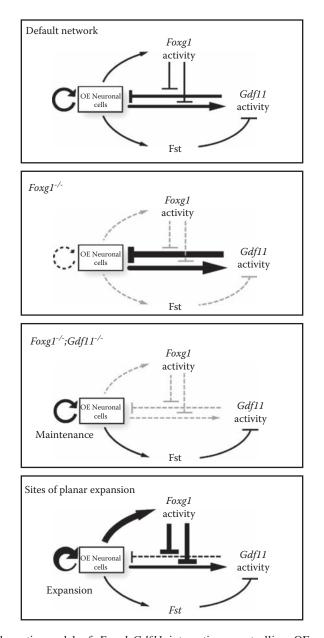
In view of the fact that FoxG1 is a transcriptional regulator, we also considered the possibility that a third mechanism—a repressive effect of FoxG1 on Gdf11 expression—might also be at play in the OE. Using quantitative RT-PCR to determine Gdf11 transcript levels in E11.5 frontonasal tissue (this age was chosen because there is still a reasonable amount of OE remaining in *Foxg1* null animals), we found that Gdf11 expression in  $Foxg1^{-/-}$  and  $Foxg1^{-/-};Gdf11^{+/tm2}$  mutants is significantly lower than that in wildtype littermates (Kawauchi et al. 2009). This is not surprising given that Gdf11 is expressed in the OE and there is substantially less OE tissue in such mutants than in wildtype animals. Indeed, Q-RT-PCR shows that levels of Sox2, a marker of OE neuroepithelial cells at this age (Figure 10.8A), are also markedly decreased in  $Foxg1^{-/-}$  and  $Foxg1^{-/-};Gdf11^{+/tm2}$  mutants. However, when Gdf11 transcript levels are normalized to Sox2 transcript levels in the same samples, to correct for the different amounts of OE in the different mutants, it was found that Gdf11 levels are actually two- to threefold higher, per amount of OE, in  $Foxg1^{-/-}$  embryos than in wildtypes. This suggests that relative increases in GDF11 activity, within what little OE remains in  $Foxg1^{-/-}$  mice, may contribute to the severity of the OE phenotype in these animals.

The observations that loss of Foxg1 results in increased GDF11 signaling, increased Gdf11 expression, and decreased expression of a GDF11 signaling antagonist (*Fst*), collectively suggest that the relationship between GDF11 and FoxG1 activity is a highly sensitive one. If, as we suggest, it is the OE itself that induces expression of *Fst* in its underlying stroma, then a positive feedback loop that controls OE neurogenesis emerges (Figure 10.10): an increase in *Gdf11* activity would lead to a decrease in OE size, which would cause a decrease in *Fst* expression, which would, in turn, cause an increase in *Gdf11* activity. A decrease in *Gdf11* activity would be similarly self-enhancing. According to this view, GDF11 in embryonic OE is less of a graded regulator of neuronal production than a switchlike controller of a self-sustaining program of neurogenesis—with FoxG1 regulating when and where the switch is thrown (Figure 10.10).

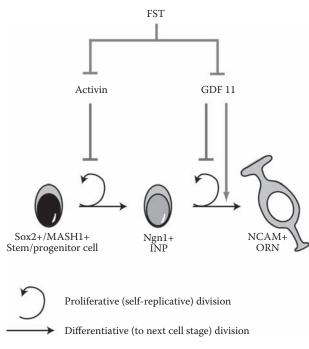
During embryonic development of the OE, the process of neurogenesis can be viewed as serving two distinct ends: (1) histogenesis, the generation of an appropriate complement and number of OE



**FIGURE 10.9** Loss of *Fst* expression in *Foxg1*- $\checkmark$  OE is rescued by inactivation of *Gdf11*. (A) Defective OE neurogenesis (histogenesis), but not nasal cavity morphogenesis, is observed in *Fst*- $\checkmark$  olfactory epithelium. ISH for OE neuronal lineage markers (*Ngn1* and *Ncam*) was performed on the OE of E17.5 wildtype and *Fst*- $\checkmark$  mutant littermates. In *Fst*- $\checkmark$  mice, the basic size and shape of the nasal cavity are the same as in wildtype, and convoluted turbinate structures are observed in the mutants. The OE of *Fst*- $\checkmark$  mice is, however, much thinner than that of wildtype littermates and is relatively devoid of *Ngn1*-expressing INPs, with a much thinner *Ncam*-expressing ORN layer (see insets). Anterior is right, posterior is left, lateral is top, the nasal septum is at the bottom of each panel. NC, nasal cavity; T, turbinate; OE, olfactory epithelium; sep, septum; scale bar: 200 µm. (B) ISH for *Fst* performed on wildtype and *Foxg1*- $\checkmark$  mice at different developmental stages. At E16.5, when *Fst* is expressed in both the OE and underlying stroma in wildtype mice (B, top right panel), it is undetectable anywhere in the nasal mucosa of *Foxg1*- $\bigstar$  embryos (in those rare instances when remnants of nasal mucosa can be observed). NE, nasal epithelium; OE, olfactory epithelium; BL, basal lamina; nc, nasal cavity; Str, stroma; fb, forebrain; scale bars: 100 µm in E10.5 and E12.5, 50 µm in E16.5. (C) *Fst* expression is restored in rescued OE (and underlying stroma) of *Gdf11*+ $\bigstar$ ;*Foxg1*- $\bigstar$  and *Gdf11*- $\div$ ;*Foxg1*- $\bigstar$  mice (*Gdf11*-ئ is used to designate the *Gdf11*<sup>m2</sup> allele in this figure). Scale bar: 50 µm. (Adapted from Kawauchi, S. et al. *Development*, 2009.)



**FIGURE 10.10** Schematic model of *Foxg1-Gdf11* interactions controlling OE neurogenesis. Default network: in wildtype OE, *Foxg1* and *Gdf11* are both produced by OE neuronal cells, but *Foxg1* proneurogenic activity antagonizes both the antineurogenic activity of *Gdf11*, and the production of *Gdf11* by OE neuronal cells. OE neuronal cells also express *Fst*, and *Fst* action antagonizes *Gdf11* activity. This default network of gene activities controls the normal steady-state level of neurogenesis in the OE. *Foxg1<sup>-/-</sup>* OE: In *Foxg1<sup>-/-</sup>* OE, *Foxg1* activity is absent, and *Fst* expression is downregulated, resulting in hypersensitivity of the OE to the action of *Gdf11*. Both OE neurogenesis and planar expansion of the OE fail. *Foxg1<sup>-/-</sup>;Gdf11<sup>-/-</sup>* double mutant OE: *Fst* expression is restored and histogenesis (neurogenesis) within the OE is rescued, since the antineurogenic activity of *Gdf11* is now removed and any similar antineurogenic factors are antagonized by *Fst*. Sites of planar expansion: *Foxg1* activity strongly inhibits both *Gdf11* activity and expression, which would allow the OE to undergo planar expansion in sites where *Foxg1* is highly expressed in wildtype OE (e.g., posterior recess of the nasal cavity). Once expansive growth is finished, *Foxg1* expression is downregulated (e.g., anterior septum), and OE neurogenesis returns to its default state. (Adapted from Kawauchi, S. et al. *Development*, 2009.)



**FIGURE 10.11** Schematic of feedback regulation of the ORN lineage. ORN production is the result of stem and progenitor cell divisions that are replicative (curved arrows) and differentiative (straight arrows). GDF11, which is produced by INPs and immature ORNs, negatively regulates replicative divisions of INPs and promotes differentiation of ORNs from INPs (Adapted from Wu, H.H. et al. *Neuron*, 37, 197–207, 2003; Lander, A.D. et al. *PLoS Biol*, 7, e15, 2009). Activin produced in the OE negatively regulates replicative divisions of *Sox2*- and *Mash1*-expressing stem/early progenitor cells (Gokoffski and Calof, unpublished observations). FST, which is synthesized in both OE and underlying stroma, antagonizes activin and GDF11 signaling (Adapted from Wu, H.H. et al. *Neuron*, 37, 197–207, 2003; Gokoffski and Calof, unpublished observations).

cells at each location along the epithelium; and (2) morphogenesis, the planar growth and invagination of the epithelium that produces the deep folds characteristic of the nasal cavity. In *Foxg1*-/embryos, both processes fail from early stages. Yet, when *Foxg1* mutants are rescued through loss of *Gdf11*, the two processes are restored to very different degrees. Histogenesis is nearly normal in *Foxg1*-/-, *Gdf11*+/*tm2* and *Foxg1*-/-;*Gdf11tm2*/*tm2* mutants; but morphogenesis is impaired in *Foxg1*-/and *Gdf11tm2*/*m2* mice, and even more so in *Foxg1*-/-;*Gdf11*+/*tm2* animals (Figure 10.8C and D).

These phenotypes may be explained by the expression pattern of Foxg1 in the developing OE: Foxg1 is initially found throughout the OE, but soon becomes localized primarily to those areas in which planar expansion of the epithelium is occurring (Figure 10.8B). This suggests that Gdf11 levels in most of the OE are normally low enough to permit a constant, steady accumulation of ORNs, leading to normal histogenesis. However, at locations where Foxg1 is strongly expressed, potent inhibition of GDF11 signaling might allow the tissue to switch into a mode of more dramatic expansion. As discussed above, Lander and colleagues have used mathematical modeling to show that the only change needed to convert a tissue that adds cells at constant rate, to one that adds cells at an exponentially increasing rate, is adjustment of the ratio of proliferative divisions vs. differentiative divisions of a stem or transit-amplifying cell to a level above 50% (Lander et al. 2009). Since GDF11 demonstrably lowers INP replication probabilities (Figure 10.5; Wu et al. 2003; Lander et al. 2009), sufficient reduction in Gdf11 activity could switch the OE into an exponential growth mode. However, in regions of Foxg1 expression, GDF11 signaling is effectively blocked (through

the cell-autonomous action of FoxG1 on GDF11 signaling). Together, these observations explain why absence of FoxG1 leads to failure of both histogenesis and morphogenesis in the OE (Figure 10.10): since unopposed GDF11 activity occurs everywhere, planar growth and neurogenesis are both halted.

#### 10.6 CONCLUDING REMARKS

As with all biological systems, the key to understanding complexity is to recognize that evolution selects for that which enhances fitness. Collectively, the work summarized here demonstrates the utility of blending experimental discoveries with computational modeling: Not only are we able to uncover the key players that participate in complex biological systems, we can also gain insight as to what such complexity achieves.

Most attempts to identify and characterize molecules that regulate neurogenesis have focused on isolating factors that promote stem cell self-renewal. These efforts have been aimed at understanding how neuronal stem cell pools (which are limited in the central nervous system) might be expanded (Lennington et al. 2003; Kawauchi et al. 2005; Nystul and Spradling 2006). However, studies such as those described in this chapter, indicate that feedback—in particular, negative feedback—of self-replication and differentiation is likely to be an especially important factor in controlling the behaviors of stem and progenitor cells. Such findings indicate that identification of such negative regulators, and understanding how they function in complex systems, will be of crucial importance for advancing our basic understanding of stem cells, and for directing their eventual use in cell replacement therapies to treat injury and disease.

#### ACKNOWLEDGMENTS

Work on this project was supported by grants from the March of Dimes Birth Defects Foundation and from the NIH (DC03583 and GM076516) to A.L.C. and to A.D.L. (GM076516). K.K.G. was supported by training grants from the NIH (NS07444 and GM08620) and the MSTP program of the University of California, Irvine.

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## 11 Neurogenesis in the Adult Olfactory Bulb

Angela Pignatelli and Ottorino Belluzzi

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## 11.1 HISTORICAL NOTE

For over a century, a central paradigm in the field of neuroscience has been that the capacity of germinal layers to generate neurons was restricted to the embryonic period, and that new neurons are not added to the adult mammalian brain (Ramon y Cajal 1913). Occasional early reports of neurogenesis in the adult central nervous system (CNS) (Allen 1912; Levi 1898) were ignored, probably because of the impossibility to determine with certainty the neuronal nature of the cells presenting mitotic figures. In more recent times, the pioneering work of Altman (1962), followed by the studies of Kaplan and Hinds (1977), have reproposed, this time with more compelling evidences, that new neurons are added in discrete regions of the adult brain, the olfactory bulb (OB) and the dentate gyrus (DG) of the hippocampus (for a historical review, see Kaplan 2001). These reports were initially ignored, then followed by negative reactions and critical publications that did not confirm the existence of newborn neurons in adults (for a review of the controversy, see Gross 2000). After the finding

that in reptiles new neurons continue to be added to most of the telencephalon throughout life (for a review, see Garcia-Verdugo et al. 2002), the paradigm shift leading to the acceptance of the notion of adult neurogenesis in higher vertebrates has known an important acceleration thanks to the discovery of neurogenesis in birds, related to the appearance of seasonal song (for review, see Nottebohm 1989). Nevertheless, these initial discoveries confronted the persistent assumption that adult neurons did not undergo proliferation, the last trench being dug at the level of the mammalian brain (Rakic 1985). The turning point of the collective perception about neurogenesis occurred with the demonstration that adult mammalian brain neurons are also capable of mitosis, and that newborn neurons can migrate and integrate into existing circuitries (for review, see Gross 2000). Interestingly, this particular new form of structural brain plasticity is specific to discrete brain regions and most investigations concern the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampus (for a review, see Lledo et al. 2006). Although, in the past, occasional reports have appeared suggesting neurogenesis also at the cortical level (Gould et al. 1999), an elegant paper appeared a few years ago (Bhardwaj et al. 2006) providing almost definite proof that, contrary to other cell types in the brain parenchyma, no new cortical neurons are generated after the perinatal period. Taking advantage of the integration of C-14 generated by nuclear bomb tests and by analyzing neocortical tissue of patients who received bromodeoxyuridine (BrdU), the study provides compelling evidence that there is no biologically significant neocortical neurogenesis in adult humans.

#### 11.2 NEUROGENESIS

#### 11.2.1 DEVELOPMENT AND ROLE OF THE SUBVENTRICULAR ZONE (SVZ)

The SVZ (Boulder Committee 1970), which develops from residual progenitors of the lateral ganglionic eminence (LGE) (Bayer et al. 1994), is one of the major germinal layers during embryogenesis, giving rise to most neurons and glial cells in the forebrain. At late stages of embryonic development, it generates cells destined for the adjacent basal ganglia and for other structures in the brain, including the diencephalon (Rakic and Sidman 1969) and cortex (Anderson et al. 1997; de Carlos et al. 1996). Contrary to other germinal layers, the SVZ persists after birth, lining most of the lateral wall of the lateral ventricle, and, together with the hilar region of the hippocampus, remains one of the two districts in the mammalian brain where neurogenesis persists throughout life (Alvarez-Buylla and Lim 2004), although, compared with the LGE from which it originates, the postnatal SVZ shows a more restricted lineage potentiality (Stenman et al. 2003; Wichterle et al. 2001).

Until recently, it was believed that the germinative zone in the adult was restricted to the wall of the lateral ventricle facing the striatum (lateral wall) in which postnatal proliferation is more easily observed. However, the rostral migratory stream (RMS) and parts of the lateral ventricular wall facing the septum (medial wall), or the corpus callosum or pallium (dorsal wall), contain proliferative cells that act as stem cells in vitro (Doetsch et al. 1999; Gritti et al. 2002) and in vivo (Merkle et al. 2007), so together these regions might be considered a unique large proliferative zone. Further findings that the region involved in adult neurogenesis in the OB might extend well beyond the SVZ comes from the work of Ventura and Goldman (2007), which provides a direct demonstration of a contribution of the dorsal radial glia, formerly believed to senesce postnatally, to the generation of interneurons in the adult OB. Nevertheless, for simplicity, in the following we will generically speak of SVZ.

As originally described by Altman (1969), SVZ stem cells give rise to neuroblasts that migrate tangentially along the RMS into the OB, where they migrate radially to complete their differentiation into different types of interneurons (Luskin 1993; see below).

#### 11.2.2 PROGRESSION OF CELL TYPES WITHIN THE ADULT SUBVENTRICULAR ZONE (SVZ)

Since the first description of neural stem cells in the adult SVZ (Reynolds and Weiss 1992; Richards et al. 1992), much work has been done to identify these stem cells, and to characterize their

progression toward the mature neuronal phenotype, and factors involved in regulating stem cell maintenance and behavior.

The identity of the stem cells in the adult SVZ has been extensively debated. The prevailing model, based on a variety of approaches, including cell lineage, heterochronic and heterotrophic transplantation, and morphological and immunocytochemistry analysis, suggests that a distinct group of astrocytes expressing glial fibrillary acidic protein (GFAP) and exhibiting certain radial glial properties, are neural stem cells that function as primary precursors in the SVZ (type B cells; Alvarez-Buylla and Lim 2004). These slowly proliferating cells, lying adjacent to the ependymal layer, were first identified as astrocytes on the basis of their morphology, ultrastructure, proliferation markers (Doetsch et al. 1999), and capability to form neurospheres giving rise both to neurons and glia (Laywell et al. 2000), an identity that has been further confirmed using a transgenic approach (Garcia et al. 2004). These multipotent neural progenitors produce clusters of rapidly dividing immature precursors (Dlx2+ transit fast amplifying, or type C cells), which, in turn, produce young polysialic acid neural cell adhesion molecule (PSA-NCAM+) neurons, also known as neuroblasts (type A cells) (Doetsch et al. 1999; Doetsch and Alvarez-Buylla 1996).

However, other adult SVZ stem/progenitor cells have been proposed, whose placement into the lineage model outlined above is still a matter of debate.

The adult derivatives of the embryonic forebrain germinal zones consist of two morphologically distinct cell layers surrounding the lateral ventricles: the ependyma and the subependyma. Ependymal cells, which form a multiciliated single cell layer lining the ventricles and are in close proximity to the cells of the SVZ, have been proposed as stem cells (Johansson et al. 1999). However, other studies have challenged this initial report, proposing the subependymal cells as stem cells (Capela and Temple 2002; Chiasson et al. 1999; Doetsch et al. 1999). A recent study reports that, in the adult mouse forebrain, immunoreactivity for a neural stem cell marker, prominin-1/CD133, is exclusively localized to the ependyma, although not all ependymal cells are CD133(+) (Coskun et al. 2008). Using transplantation and genetic lineage tracing approaches, these authors demonstrate that CD133(+) ependymal cells continuously produce new neurons destined for the OB, and propose that these cells may represent an additional—perhaps more quiescent—stem cell population in the mammalian forebrain, which add to the GFAP+ adult neural stem cells (Coskun et al. 2008).

Taken together, these findings emphasize the complexity of the issue about the identity of neural stem cells in vivo, and call for further investigations to tie up the many loose ends, for example, the placement of LeX+ (Aguirre et al. 2004; Capela and Temple 2002; Platel et al. 2009) or nestin+ (Beech et al. 2004; Burns et al. 2009; Lagace et al. 2007) adult SVZ stem/progenitor cells into the current lineage model. Conceivably, there might be several sources of neural stem cells in the adult SVZ that might get involved in different situations, as occurs in the adult olfactory epithelium, where distinct cell populations mediate normal neuronal turnover and neuronal replacement under special circumstances (Leung et al. 2007).

#### 11.2.3 **R**EGIONALIZATION OF NEURONAL STEM CELLS

Neuronal progenitors bound to the OB originate from all rostrocaudal sectors of the SVZ (Doetsch and Alvarez-Buylla 1996; Kirschenbaum and Goldman 1995). It has long been held that neural stem cells in the SVZ are a homogeneous population of multipotent, plastic progenitors, and that neuroblasts born in the SVZ might be equivalent until they reach the OB and begin to differentiate. However, it has first been shown that a certain degree of molecular heterogeneity already exists in migrating SVZ neuroblasts before reaching the OB (Baker et al. 2001; De Marchis et al. 2004; Jankovski and Sotelo 1996), and then that neural stem cells in the SVZ are organized in a multiple restricted and diverse population of progenitors (Beech et al. 2004; Hack et al. 2005; Kelsch et al. 2007; Kohwi et al. 2005; Merkle et al. 2007; Waclaw et al. 2006; Young et al. 2007).

By taking advantage of the regionally restricted embryonic expression of different transcription factors (Kriegstein and Gotz 2003), transgenic mice (Nkx2.1-Cre, Gsh2-Cre, Emx1-Cre, Dbx1-Cre, and Emx1-CreER<sup>T2</sup>) were crossed with Cre reporter mice in fate-mapping experiments (Kohwi et al. 2007; Young et al. 2007). BrdU and staining for cell-type-specific markers was used to identify adult-generated cells. Descendants of the embryonic LGE and cortex settle in ventral and dorsal aspects of the dorsolateral SVZ, respectively. Both generate RMS neuroblasts and are responsible for generating olfactory interneurons throughout life. However, these two stem cell populations make unequal contributions to adult neurogenesis. Cortex-derived stem cells (Emx1+), generate primarily calretinin-positive (CalR+) and tyrosine hydroxylase (TH)+ periglomerular (PG) cells, but none of the calbindin-positive (CalB+) interneurons. LGE-derived stem cells (Gsh2+) generate all of the adult-born CalB+ interneurons for the olfactory glomerulus (Kohwi et al. 2007; Young et al. 2007).

In a systematic study carried out by Merkle et al. (2007), the radial glia was labeled in a regionally specific manner by stereotaxical injection of small volumes of adenovirus-expressing Crerecombinase in neonatal (P0) LacZ/eGFPG reporter mice (Novak et al. 2000). Injected mice were then analyzed 4 weeks later and labeled OB interneurons were stained for cell-type-specific markers (Kosaka et al. 1995). Fifteen different populations of neuronal stem cells were targeted in the SVZ at different rostrocaudal and dorsoventral levels, including the RMS, the medial (septal) wall, and the cortical wall of the lateral ventricle. They found that OB interneurons are produced from the entire SVZ, including regions of the cortical walls located beyond the accepted boundary of the adult neurogenic zone. However, each region gives rise to only a very specific subset of interneuron subtypes. This is particularly evident for PG cells that, on the basis of their neurochemical properties, can be subdivided into nonoverlapping populations subserving different functions in the bulbar circuitry, basically calretinin- and calbindin-expressing cells, and dopaminergic (DA) cells (Kosaka et al. 1995; Parrish-Aungst et al. 2007). Anterior and dorsal regions produce PG cells in a regionspecific manner. So, for example, DA neurons and CalB+ cells originate from stem cells located in the dorsal and ventral regions, respectively. An analogous regionalization is also observed for granule cell precursors: each targeted region produces granule cells, but dorsal regions tend to produce superficial granule cells, whereas ventral regions produce mostly deep granule cells. Finally, CalR+ cells, either PG or granule cells, originate in the same areas, RMS and medial (septal) wall. Interestingly, the site of origin within the adult SVZ not only determines the specific markers and final position of postnatally generated interneurons within the OB, but also the specific projection of their dendrites (Kelsch et al. 2007); see below.

It is of some interest to observe how stem cells colonizing different parts of the SVZ and generating different neuronal progeny, have different embryonic origins, suggesting that some characteristic of embryonic patterning is maintained in the adult SVZ. Under this aspect, the diversity of adult-generated bulbar interneurons seems to originate from a process more akin to that of cortical interneurons, deriving from distinct progenitor pools (Wonders and Anderson 2006), rather than to that of cerebellar interneurons, originating from multipotent precursors that acquire their mature identities under the influence of local instructive cues (Leto et al. 2006).

## 11.2.4 TIMING

Neonatal and adult SVZ progenitors differentially contribute to neurochemically and functionally distinct types of interneurons following a precise timing.

In one study, mice were given a single pulse of BrdU at different time points, and BrdU-labeled nuclei in the granule cell layer (GrL) were quantified after 20–28 days (Lemasson et al. 2005). Cells labeled at P3 or P7 were more likely to integrate in the superficial GrL and survive, compared to cells born at later ages.

Another group injected the SVZ with dye or grafted it at different ages and suggested that different subtypes of PG cells might be preferentially produced at different ages (De Marchis et al. 2007). Labeled PG cells are more likely to be CalB+ if derived from the neonate, and more likely to be CalR+ or TH+ if derived from the adult-labeled SVZ. Also, embryonic or neonatal cells grafted into the neonatal or adult brain produce different cell types: again, younger tissues produce a higher percentage of CalB+ cells and a lower percentage of CalR+ cells than older tissues. Some of these findings have received further support from a more recent study (Batista-Brito et al. 2008).

Taken together, apart from some difference between these studies, it is becoming clear that different cell types are preferentially produced at different ages. This might be relevant because, since these different cell types integrate into different OB circuits, the temporal pattern of their production might regulate the functional maturation of the OB.

## 11.2.5 FACTORS REGULATING ADULT NEUROGENESIS

Neuronal stem cells' self-renewal and differentiation are regulated by a specialized microenvironment conventionally referred to as the germinal niche—in which these cells reside (Doetsch 2003; Moore and Lemischka 2006). A large assortment of intrinsic genetic programs (Hack et al. 2005; Kohwi et al. 2005; Waclaw et al. 2006) and extrinsic environmental cues (Hack et al. 2005) direct or regulate the balance of self-renewal and differentiation in all stem cells within niches and on their way to the OB. Stem cells, their progeny, and elements of their microenvironment make-up an anatomical structure that coordinates normal homeostatic production of functional mature cells.

## 11.2.5.1 Cellular Niches

In vertebrates, adult-born neurons are the progeny of precursor cells residing within specialized brain regions, termed neurogenic niches (Doetsch 2003; Garcia-Verdugo et al. 2002; Ma et al. 2005); for a review see Moore and Lemischka (2006). In ecology, a niche is a term describing the relational position of an organism or population in its ecosystem, what it does, and how it interacts with its close environment. Accordingly, a neurogenic niche is an interactive structural unit, organized to facilitate the complex local interactions occurring between neuronal stem cells and their close environment, in order to produce cell-fate decisions in a proper spatiotemporal manner. The cellular and extracellular elements that make up neurogenic niches not only support the precursor cells structurally, but also functionally regulate their activity and the development of their progeny (Doetsch 2003; Ma et al. 2005; Shen et al. 2004; Song et al. 2002). Glial cells are key components of the neurogenic niches of adult vertebrates, acting both as the precursor cells and in the support and regulation of neurogenesis (Doetsch 2003; Garcia et al. 2004; Garcia-Verdugo et al. 2002; Ma et al. 2005; Seri et al. 2004; Song et al. 2002). These cells also guide and regulate the migration of newborn cells to the regions of the brain in which they differentiate into neurons (Bolteus and Bordey 2004; Lois et al. 1996). Additional important niche elements include a close association with the vasculature and the presence of specialized basal lamiae (Doetsch 2003; Garcia-Verdugo et al. 2002; Ma et al. 2005; Mercier et al. 2002; Palmer et al. 2000, 2002).

Striking similarities have been described between adult neurogenesis in the invertebrate brain (in freshwater crayfish) and what is known about the origin of new neurons in adult vertebrate brain (Sullivan et al. 2007). In the adult avian and mammalian brain, the precursor cells reside within a specialized niche supporting self-renewal and differentiation. Precursor cells for adult neurogenesis in crayfish are also glial cells that reside within a niche containing specialized basal lamina and vasculature (Sullivan et al. 2007). Furthermore, like neurogenic astrocytes in the mammalian brain, these glial cells appear to function not only as precursors, but also as support cells to guide the directional migration of neuroblasts. As has been observed (Alvarez-Buylla 2007), it is intriguing that common strategies are used across such phylogenetically distant species, and it will be interesting to widen the range of species studied to understand whether these similarities are a result of a common evolutionary origin for adult neural stem cells or of convergence.

#### 11.2.5.2 Intrinsic Factors

Intrinsic factors can be defined as the ensembles of signals expressed by stem cells and progenitors that control different neurogenic phases, as opposed to external factors, which are produced by

surrounding tissues to act on stem cells and progenitors. Intrinsic factors can be phenotypic-independent or phenotypic-specific. A list of intrinsic factors is shown in Table 11.1.

## 11.2.5.3 Extrinsic and Epigenetic Factors

The processes of newborn neuron proliferation, migration, maturation, targeting, and survival are all subject to modulation by environmental signals, like neurotransmitters, growth factors, hormones, and a variety of environmental factors, including various injuries, summarized in Table 11.2. Also a complement of epigenetic factors, including mitogenic or antiproliferative factors in the local environment, have been shown to control the duration of the cell cycle or the number of cells cycling and the speed of neuroblasts migration prior to their integration into the OB circuitry (for reviews, see Bordey 2006; Hagg 2005).

For the near future, it will be worth keeping an eye on recent patents concerning novel small molecules, identified from screening collections, which would stimulate or otherwise regulate stem cell differentiation and neurogenesis. Several recent patents claim newly discovered neural stem cells differentiation modulating the activity of previously marketed drugs, suggesting perhaps a previously unknown mechanism of action of these drugs and/or implicating the target enzyme and receptor pathways as key players in neurogenesis (Rishton 2008).

## 11.2.6 THE SUBVENTRICULAR ZONE (SVZ) IN HUMANS

Although, to date, the notion of an active neurogenesis from neural progenitors continuing throughout life in discrete regions of the CNS of mammals can be considered as firmly established, the point at which neurogenesis studies can be extrapolated to humans is still a matter for discussion (Breunig et al. 2007).

In humans, as in rodents, the SVZ contains cells that proliferate in vivo, and behave as multipotent progenitor cells in vitro (Bédard and Parent 2004; Bernier et al. 2000; Johansson et al. 1999; Kirschenbaum et al. 1994; Kukekov et al. 1999; Nunes et al. 2003; Palmer et al. 2001; Roy et al. 2000; Sanai et al. 2004). The existence of the equivalent of the SVZ-RMS was reported also in primates (macaque; Kornack and Rakic 2001), and it was therefore with some surprise that, in 2004, an investigation based on a large number of postmortem and biopsy samples reported that the RMS—or an equivalent structure—was missing in humans (Sanai et al. 2004; see also comment by Rakic 2004), an observations that seemed to confirm a previous report showing that migratory neuronal precursors are present in humans during infancy, but seem to disappear during childhood (Weickert et al. 2000).

A more recent report, however, realigns findings from rodents concerning the potential for neurogenesis in the adult mammalian brain with human structures (Curtis et al. 2007). For its importance, this paper deserves some space in this context. Briefly, the authors, as in the study of Sanai, have obtained postmortem samples of the adult human brain, and used a combination of basic and specific stains to locate and identify the complex SVZ-RMS-OB in adult humans, and to characterize the cells within the area. The work first illustrates, through proliferating cell nuclear antigen (PCNA is a marker for proliferating cells) staining and a Nissl counterstain, the presence of an RMS-like pathway between the cerebral ventricles and the OB; this network of cells streams in a caudal-ventral direction, before turning rostral along the olfactory tract toward the OBs. Next, ultrastructural studies verify that the human SVZ and all levels of the RMS contain cells with migratory-like (type A) morphology, and stain positive for PSA-NCAM,  $\beta$  III-tubulin, and doublecortin. The authors further show a gradient of expression of markers of cell differentiation (Pax6, Olig2, and DCX) all along the pathway from the cerebral ventricles to the OB. Next, the work shows that the human RMS is organized around a tubular extension of the lateral ventricle that reaches the OB via cross sections from postmortem human tissue and MRI scans of the forebrain/OB region. Finally, using double staining for BrdU and NeuN, they show that progenitor cells become mature neurons in the OB.

Certainly, caution must be used in evaluating all these data, as authoritatively and appropriately reminded (Breunig et al. 2007; Rakic 2002), but, on the basis of the experimental evidence

TABLE 11.1 Intrinsic Factors	TABLE 11.1 Intrinsic Factors Involved in Neurogenesis and Morphogenesis in the SVZ	esis in the SVZ
Acronym	Nature	Description
Aβ	Amyloid beta—peptide of 39–43 amino acids formed after sequential cleavage of the amyloid precursor protein, a transmembrane glycoprotein of undetermined function	$A\beta(25-35)$ can convert nestin-negative precursors toward nestin-positive neural precursor cells, and nestin-positive NPCs into neuroblasts. Intracerebroventricular infusion of $A\beta(1-42)$ increases the population of PSA-NCAM-positive cells in the SVZ, without affecting proliferation (Calafiore et al. 2006)
AP-1	Activator protein 1—transcription factor; heterodimeric protein composed of proteins belonging to the c-Fos. c-Jun, ATF, and JDP families	c-Fos, Fos-B, Jun-D are colocalized with TH+ PG neurones (Liu et al. 1999). AP-1 is implicated in the regulation of TH expression in the olfactory bulb dopaminergic neurons (Baker et al. 2001; Liu et al. 1999)
Arx	Aristaless-related homeobox-homeodomain transcription factor	Expressed in the embryonic LGE as well as the postnatal SVZ, RMS, and OB (Colombo et al. 2007; Yoshihara et al. 2005); Arx-deficient mice show size reduction of the OB, reduced proliferation OB interneuron progenitors, loss of TH+ PG cells, disorganization of the layer structure of the OB, and abnormal axonal termination of olfactory sensory neurons (Yoshihara et al. 2005)
ASCL1	Achaete-scute complex-like 1	a.k.a. MASHI - see below
CRE and TPE	(cAMP response element and TPA responsive element) are transcription factor	Colocalized with TH in the adult-born dopaminergic periglomerular neurons (Liu et al. 1999). Expressed by the SVZ neuroblasts; transgenic mice lacking CREB show reduced survival of newborn neurons in the OB (Giachino et al. 2005)
Doublecortin (DCX)	Microtubule-binding protein	Expressed in migrating neuroblasts in the postnatal SVZ, RMS, and OB (Brown et al. 2003; Gleeson et al. 1999; Nacher et al. 2001); required for nuclear translocation and maintenance of bipolar morphology during neuroblasts migration (Koizumi et al. 2006; Ocbina et al. 2006), but see (Nam et al. 2007)
DLX 1-2	Family of distal-less related homeobox proteins; transcription factors	Expressed in M- and LGE (Bulfone et al. 1993; Saino-Saito et al. 2003), adult SVZ (Liu et al. 1997; Porteus et al. 1994; Stemman et al. 2003), RMS (Long et al. 2007), and OB. Regulate several molecular pathways, are required for the generation of OB neuroblasts, and are required for promoting tangential misration to the OB (Long et al. 2007)
DLX 5-6	Family of distal-less related homeobox proteins; transcription factors	Expressed in the SVZ (Liu et al. 1997) and OB (Long et al. 2003). D1x5 affects the ability of neural progenitors to produce OB local circuit neurons, with granule cells more affected than PG cells (Long et al. 2003)
E2F1	Member of E2F family of transcription factors	E2F1-deficient mice show a lower level of cell proliferation and a reduction in the number of neurons generated in adult neurogenic areas (Cooper-Kuhn et al. 2002)
Emx2	Empty spiracles homeobox 2; homeodomain transcription factor	Controls rate of proliferation—expressed by MSC in periventricular region (Galli et al. 2002)

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(Continued)	
<b>VBLE 11.1</b>	
TABL	

Acronym	Nature	
Ephrins	Membrane-bound ligands for Eph receptor tyrosine kinases (Klein 2008)	Бран
Er81	Protein belonging to the ETS class of DNA-binding transcription factors	Ϋ́ Ϋ́ Ϋ́
Gsh2	Homeobox transcription factor	O EX C
Id2	Inhibitor of DNA-binding 2	ъ е De a
Mashl	Transcription factor of the basic helix-loop-helix (bHLH) family	S
Meis2	Homeobox protein belonging to the TALE ("three amino acid loop extension") family. It is a transcription regulator	N N
Neuregulins (NRG)	Ligand of ErbB4 receptor tyrosine kinase	Ϊ
Notch1	Single pass transmembrane receptor for membrane- bound ligands	o T c
		20002
Numb and	Membrane-bound proteins; intrinsic Notch regulatory	re Re

# Description

phrins A2, B2, and B3 and Eph receptors A4, A7, and B1-3 are expressed in the postnatal SVZ and RMS where they regulate progenitor proliferation and migration (Conover et al. 2000; Holmberg et al. 2005; Ricard et al. 2006; Zimmer et al. 2008)

r81 is expressed in the embryonic dLGE and the postnatal SVZ, RMS, and OB (Saino-Saito et al. 2007; Stemman et al. 2003). ER81 is expressed in dopaminergic cells and in a subset of CalR+ cells in the GL Allen et al. 2007) in activity-dependent manner (Saino-Saito et al. 2007)

xpressed in the embryonic LGE and postnatal SVZ, RMS, and OB (Carney et al. 2009; Parmar et al. 2003; Toresson and Campbell 2001); are downstream target of Shh (Corbin et al. 2000); Gsh2(-/-) embryos suffer from an early misspecification of precursors in the LGE leading to disruptions in striatal and OB development (Yun et al. 2003)

eletion reduces numbers of granular and periglomerular neurons with a distinct paucity of dopaminergic PG neurons (Havrda et al. 2008) jives rise to either neurons (Neurogenin2-positive) or oligodendrocytes (Olig2-positive) also in postnatal SVZ (Parras et al. 2004); considered a marker for transit-amplifying (type C) cells

feis2 is expressed in the embryonic LGE and in the postnatal SVZ (Pennartz et al. 2004), RMS and OB, where it is found in TH+, CalB+, and CalR+ cells in the glomerular layer (Allen et al. 2007)

bifferent NRG ligands affect distinct populations of differentiating neural precursors in the SVZ; NRG-ErbB4 interactions influence the proliferation and organization of cells in the SVZ (Ghashghaci et al. 2006) he Notch signaling pathway controls differentiation of cells by intercellular communication between neighboring cells and is associated with the maintenance of a progenitor, proliferative state. Notchl is associated with cells in the SVZ, RMS, and OB in the postnatal brain (Givogri et al. 2006; Stump et al. 2002); precursors of the olfactory bulb respond to Notch signals by remaining quiescent and failing to give rise to differentiated progeny of any type (Chambers et al. 2001); Notchl cascade is activated by cellin in a still unidentified way (Hashimoto-Torii et al. 2008; Keilani and Sugaya 2008). For a recent eview revising the classical role of this signal see Kageyama et al. (2008)

cegulate ependymal wall integrity and postnatal SVZ neuroblasts survival (Kuo et al. 2006); control the polarity of the neuronal progenitors in the SVZ through regulation of radial glial cell adherens junctions (Rasin et al. 2007)

proteins

Numblike

Nurr1 Olig2	Nuclear receptor-related 1 protein, member of the nuclear receptor family of intracellular transcription factors Oligodendrocyte lineage transcription factor 2	Plays a direct role for specification of DA phenotype by activating TH gene transcription in a cell context-dependent manner (Kim et al. 2003; Sakurada et al. 1999); induces the DA phenotype in cells isolated from the SVZ (Shim et al. 2007); plays a role in maintenance of TH gene expression in adult DA PG cells (Saino-Saito et al. 2004) Specifies transit-amplifying precursor fate and opposes the neurogenic role of Pax6 (Buffo et al. 2005; Use transt-root)
PK2	Prokyneticin 2cysteine-rich secreted protein	Functions as a chemoattractant for SVZ-derived neuroblasts; within the OB, it may also act as a detachment signal for chain-migrating progenitors arriving from the RMS (Ng et al. 2005); its expression is regulated by Dlx 1 and 2 (Long et al. 2007); PK2 gene is a functional target gene of proneural basic helix-loop-helix (AHT H) forcome et al. 2007).
PSA-NCAM	Polysialic acid (PSA), a posttranslational modification of neural cell adhesion molecule (NCAM); emonhilic-hinding olycomotein	PSA-NCAM is expressed on the surface of neurons and glia; inactivation of the N-CAM gene in mice results in size reduction of the OB (Cremer et al. 1994); NCAM signals direct the differentiation of SVZ-derived mecursors toward the CalR+ nhenchyne (Rockle et al. 2008)
Pax-6	From <i>Paired box gene</i> 6; Pax-6 protein is a highly conserved transcription factor	Expressed by most proliferating adult SVZ progenitors (Kohwi et al. 2005) and TH+ cells in adult OB (Dellovade et al. 1998; Stoykova et al. 1996; Stoykova and Gruss 1994); has a dual role in generating neuronal progenitors and also in directing them toward a DA PG phenotype in adult mice (Hack et al. 2005; Kohwi et al. 2005)
Ptx3	Pentraxin-related transcription factor	Neuronal activity-regulated pentraxin (Narp) mRNA is found in postnatal PG and granule cells (Shu et al. 2001); Ptx3 is required for the DA final determination (Perrone-Capano and di Porzio 2000)
Reelin	Product of the <i>reeler</i> gene, is an extracellular matrix glycoprotein	It acts as a detachment signal of neurons migrating from the SVZ (Hack et al. 2002) via ERK activation (Simó et al. 2007), possibly acting in association with the Notch signaling system (Hashimoto-Torii et al. 2008; Keilani and Sugava 2008)
SDF-1 (CXCL12)	Stromal-derived factor 1—small cytokine belonging to the chemokine family, a.k.a. chemokine (C-X-C motif) ligand 12 (CXCL12)	Present in embryonic and adult SVZ (Tiveron et al. 2006; Tran et al. 2004); may be of general importance in control of progenitor cell migration in embryonic and adult brain (Schonemeier et al. 2008; Tran et al. 2004, 2007)
SHH	Sonic hedgehog-archetypal morphogen protein (Wolpert 1969)	SHH signaling in the postnatal SVZ is required for the maintenance of the B and C cell populations and indirectly for the migration of the neurons that are generated from the adult stem cell niche (Angot et al. 2008; Balordi and Fishell 2007; Machold et al. 2003; McMahon et al. 2003; Palma et al. 2005)
Slit1 and 2	Secreted ligand proteins that bind the roundabout (Robo) receptors	Robol and 2 receptors are expressed in the postnatal SVZ and RMS (Hu 1999; Li et al. 1999; Marillat et al. 2002; Nguyen-Ba-Charvet et al. 2004; Wu et al. 1999). Their expression is regulated by Dlx 1 and 2 (Long et al. 2007). Slit proteins are chemorepellants that guide migrating SVZ-derived progenitors (Chen et al. 2001; Hu 1999; Marillat et al 2002; Sawamoto et al. 2006; Wu et al. 1999)

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	TABLE 11.1 (Continued)	
Acronym	Nature	Description
SP8	Transcription factor—member of specificity protein family—has a buttonhead box and three C2H2 zinc-fingers	Expressed in neurogenic regions giving rise to embryonic and postnatal OB interneurons; remains expressed in the CalR+ and GABAergic/non-DA interneurons of the GL; contributes to OB interneuron diversity by regulating the survival, migration, and molecular specification of neuroblasts/interneurons (Waclaw et al. 2006)
Tenascin R	Extracellular matrix glycoprotein	Contributes to the generation of a stem cell "niche" within the SVZ, orchestrates growth factor signaling in order to accelerate neural stem cell development (Garcion et al. 2004); initiates both the detachment of neuroblasts from chains and their radial migration (Peretto et al. 2005; Saghatelyan et al. 2004); grafting of tenascin-R-transfected cells into non-neurogenic regions reroutes migrating neuroblasts toward these provine et al. 2004.
TLX	Tail-less (tlx) orphan receptor	The second compared on 2007) The second provides the second has been identified so far, exclusively expressed in a subpopulation of neural stem cells in the SVZ; depletion of the leads to complete loss of neurogenesis in the adult brain (1 in et al. 2008)
Vax-1	Ventral anterior homeobox 1-homeodomain transcription factor	Expressed in the embryonic LGE 182 as well as the postnatal SVZ and RMS; the SVZ of Vax1( $-$ ) brains is hyperplastic and mostly disorganized, and the RMS is missing (Soria et al. 2004)
Zic1,3	C2H2-type zinc finger proteins-transcription factors	Expressed in the medial structures, including the septum and choroid plexus, as well as the glomerular and granule cell layers of the postnatal OB; maintain neural precursor cells in an undifferentiated state; their absence cause hypoplasia of OB (Inoue et al. 2007)

5-HT-serotonin GABA γ-amino butyric acid	Acting through 5-HT <sub>2C</sub> and 5-HT <sub>1A</sub> GABA plays well-documented role: neuroblasts release GABA which, and Bordey 2004; Liu et al. 2005);	Acting through 5-HT <sub>2C</sub> and 5-HT <sub>1A</sub> receptors in the SVZ (Banasr et al. 2004) upregulates cell proliferation (Brezun and Daszuta 1999) GABA plays well-documented roles in regulating proliferation, migration, and gene expression in neural progenitors (Platel et al. 2008). In the SVZ, newborn neuroblasts release GABA which, acting on A-type receptors, reduces the proliferation of stem cells—and thereby neurogenesis—by a nonsynaptic mechanism (Bolteus and Bordey 2004; Liu et al. 2005); ambient GABA clearance in the SVZ occurs via two GABA transporter subtypes (Platel et al. 2007)
Glutamate	Glutamate modulates adult neurogen the SVZ (Brazel et al. 2005). mGlu of neuronal-restricted precursors, v	Glutamate modulates adult neurogenesis (reviewed in Platel et al. 2008; Schlett 2006), essentially enhancing survival and proliferation of neural progenitors derived from the SVZ (Brazel et al. 2005). mGlu5 receptors are expressed in the SVZ (Di Giorgi Gerevini et al. 2004; Platel et al. 2008) and their activation might support the survival of neuronal-restricted precursors, whereas endogenous activation of mGlu1 receptors might sustain the proliferation of earlier progenitors (Castiglione et al. 2008)
NO Nitric oxide	Nutrigenic neurons have been report migration in the SVZ (Cui et al. 20 Lopez et al. 2004; Packer et al. 200	Nutrigeme neurons have been reported in the SVZ, RMS, and OB of adult mice (Moreno-Lopez et al. 2000); administration of a NO donor to adult rats stimulates migration in the SVZ (Cui et al. 2009; Zhang et al. 2001); the effect on neurogenesis is controversial, but the prevailing idea is that it has an inhibitory effect (Moreno- Lopez et al. 2004; Packer et al. 2003; Torroglosa et al. 2007)
Opioids	The effect of opioids has been studi neurogenesis (Eisch et al. 2000; Pe δ-, and/or κ-receptor immunoreact	The effect of opioids has been studied mainly in the adult hippocampus, where the activation of the opioid-signaling system by morphine and heroin decreases neurogenesis (Eisch et al. 2000; Persson et al. 2003). In the adult mice SVZ, some subpopulations of neurons, astrocytes, and oligodendrocytes differentially express μ-, δ-, and/or κ-receptor immunoreactivity, and morphine significantly decreases the number of BrdU+ GFAP(+) cells (Stiene-Martin et al. 2001)
Cannabinoids	In the postnatal SVZ ependymocytes, radial glia, and astrocytes expressing PSA-NCAM; CB1 activation increases the number cell adhesion molecule expression (Arévalo-Martin et al. 2007	In the postnatal SVZ ependymocytes, radial glia, and astrocytes express cannabinoid receptor 1 (CB1), whereas cannabinoid receptor 2 (CB2) is found in cells expressing PSA-NCAM; CB1 activation increases the number of Olig2-positive cells in the dorsolateral SVZ, whereas CB2 activation increases polysialylated neural cell adhesion molecule expression (Arévalo-Martin et al. 2007)
BDNF	Brain-derived neurotrophic factor	<b>Trophic Factors</b> Infusion of BDNF in lateral ventricles doubles the number of BrdU+ neurons in the OB (Zigova et al. 1998); not supported by a recent investigation (Galvão et al. 2008)
CNTF	Ciliary neurotrophic factor	Infusion of CNTF in lateral ventricles increases the number of BrdU+ neurons in the SVZ and enhances the migration toward the OB (Emsley and Hagg 2003); the mechanism would involve glycoprotein 130 activation, which, in turn, would activate Notch1 expression (Chojnacki et al. 2003)
HB-EGF	Heparin-binding epidermal growth factor-like growth factor	Intracerebroventricular or nasal administration increases BrdU incorporation in SVZ (Jin et al. 2003)
EGF2	Epidermal growth factor 2	Intracerebroventricular administration expands the progenitor cell population in the SVZ, increasing the number of newborn glia, but <i>reducing</i> the number of newborn neurons in the OB (Kuhn et al. 1997)
FGF2	Fibroblast growth factor 2	Intracerebroventricular or nasal administration increases BrdU incorporation in SVZ (Jin et al. 2003) and the number of newborn cells in the OB (Kuhn et al. 1997)

(Continued)

TABLE 11.2	(Continued)	
GDNF	Glial cell line-derived neurotrophic factor	Intracerebroventricular administration in transient middle cerebral artery occlusion rats doubles the number of BrdU+ cells in the SVZ (Dempsey et al. 2003)
IGF-1	Insulin-like growth factor-1	IGF-1 mRNA is expressed in postnatal SVZ (Bartlett et al. 1992); mediator of focal ischaemia-induced neural progenitor proliferation in the SVZ (Dempsey et al. 2003; Yan et al. 2006)
TGF-b1	Transforming growth factor b1	Intranasal administration enhances the number of BrdU+ cells in the SVZ and BrdU+ cells coexpressing DCX and NeuN along the RMS (Ma et al. 2008)
VEGF	Vascular endothelial growth factor	Expressed in astrocytes of the neurogenic SVZ and RMS (Balenci et al. 2007); ICV administration of VEGF-B increases the number of BrdU+ cells in SVZ; VEGFB-KO mice show impaired neurogenesis, restored by ICV addition of VEGFB (Sun et al. 2006); enhances postischaemic neurogenesis and neuromigration (Wang et al. 2007)
PDGF	Platelet-derived growth factor	PDGF is required for oligodendrogenesis, but not neurogenesis: infusion of PDGF alone is sufficient to arrest neuroblast production and induce SVZ B cell proliferation (Jackson et al. 2006)
		Hormones
Prolactin	Stimulates the production of neurc	Stimulates the production of neuronal progenitors in the SVZ of female mice during pregnancy (Shingo et al. 2003)
Thyroid hormones	Thyroid hormones stimulate the ge	generation of new cells in the SVZ (Fernandez et al. 2004) acting via an $\alpha$ receptor (Lemkine et al. 2005)
Neurosteroids	Pregnenolone-S stimulates neurog	Pregnenolone-S stimulates neurogenesis in the hippocampus (Mayo et al. 2005); no data so far for SVZ; for a recent review see Charalampopoulos et al. (2008)
Neuropeptides	At least two neuropeptides have a j (Mercer et al. 2004) and neuropej	At least two neuropeptides have a proneurogenic effect in the adult SVZ: pituitary adenylate cyclase-activating polypeptide (PACAP), acting on PAC1 receptors (Mercer et al. 2004) and neuropeptide Y (NPY) via Y1 receptors (Agasse et al. 2008)
		Other Environmental Factors
Age	Aging decreases neurogenesis in S	Aging decreases neurogenesis in SVZ (Leuner et al. 2007; Tanaka et al. 2007)
Antidepressants	Antidepressant drugs fluoxetine an antidepressants could also imply	ntidepressant drugs fluoxetine and imipramine, serotonin reuptake inhibitors, upregulate neurogenesis (Hitoshi et al. 2007); the mechanism of action of antidepressants could also imply VEGF (Warner-Schmidt and Duman 2007)
Odors	Odor deprivation following naris c stimulates neurogenesis (Rochefo	Odor deprivation following naris occlusion impairs neurogenesis in the OB (Corotto et al. 1994; Mandairon et al. 2006), and, conversely, odor-enriched environment stimulates neurogenesis (Rochefort et al. 2002). Is it through an epigenetic-promoted DNA demethylation? (Ma et al. 2009)
Sexual signals	Male golden hamsters exposed to	to an estrous female have a significant increase of BrdU+ cells in both main and accessory OB (Huang and Bittman 2002)
201655	Following acute stroke there is a d	CHIOME SUCES DECREASES WE HUMPED OF ITENTIAL STATE (THEOSHIP & ALLOW) FOLLOWING SUCES DECREASES WE HUMPED OF ITENTIAL STATE (THEOSHIP & ALLOWING ACTES AND ALLOWING A
Stroke	glial cells (Zhang et al. 2007); mi in these conditions (Thored et al.	glial cells (Zhang et al. 2007); microglia with proneurogenic phenotype accumulates in the SVZ after stroke, implying a supportive role of these cells for neurogenesis in these conditions (Thored et al. 2008); increase of neurogenesis following stroke has also been shown in humans (Macas et al. 2006)

available to date, it seems not inappropriate to conclude that neurogenesis does exists in the human SVZ, producing new neurons to the OB following modalities that are similar to those described in rodents, and equally robust in quantitative terms.

## 11.3 MIGRATION

Neuroblasts born in the SVZ migrate to the OB where they differentiate into local interneurons (Altman 1969; Belluzzi et al. 2003; Carleton et al. 2003; Lois and Alvarez-Buylla 1994; Luskin 1993). The neuroblasts migrate within the rostral extension of the SVZ along the RMS within tubelike structures formed of GFAP-positive astrocytes. These glial cells, all along the RMS and up to the OB, possess neurogenic potential themselves: multipotential (neuronal-astroglial-oligodendroglial) precursors with stem cell features have been isolated not only from the SVZ, but also from the entire rostral extension, including the distal portion within the OB (Aguirre and Gallo 2004; Gritti et al. 2002). Stem cells isolated from the proximal RMS generate significantly more oligodendrocytes than neurons or astrocytes, and those from the distal RMS proliferate significantly more slowly than stem cells derived from the SVZ and other RMS regions (Gritti et al. 2002).

A recent paper shows that there are significant differences at the translational level between neural progenitor cells from SVZ and RMS/OB (Maurer et al. 2008). Protein expression profiles differ not only in the quantity of single proteins (more numerous in SVZ vs OB), but also in their quality: some protein species are expressed in only one of the two groups (e.g., in the OB proteins involved in differentiation and microenvironmental integration, in SVZ GFAP), others in both groups (neuronal progenitor cell marker nestin, and the mature neuronal markers, Tubulin- $\beta$ -III). A possible explanation is that microenvironmental stimuli, such as growth factors, neurotransmitters, and cell surface molecules, influence the proteome in a spatial and temporally restricted manner (Maurer et al. 2008).

Neuroblasts migration is a critical event in the process of adult neurogenesis, and perhaps one of the most complex and far-reaching forms of neuronal migration. In rodents, newborn neurons first migrate in the SVZ, and then join the RMS, which leads them into the core of the OB. The newly generated cells migrate rostrally, up to 5 mm in rodents and up to 20 mm in monkeys, to reach the OB (Doetsch and Alvarez-Buylla 1996; Kornack and Rakic 2001; Lois and Alvarez-Buylla 1994). This migration follows, without dispersion, the RMS, and requires 4–10 days in rodents (Hu et al. 1996; Lois and Alvarez-Buylla 1994; Luskin 1993; Winner et al. 2002). The dynamic analysis of the migratory process, realized with time-lapse videomicrography, revealed that individual cells migrate very rapidly, from 30  $\mu$ m/h (personal unpublished observation) to 122  $\mu$ m/h (Wichterle et al. 1997). A number of factors are known to regulate this process (see below).

Although in normal conditions the migrating neuroblasts are directed only to the OB, it has been shown that after lesions to the cerebral cortex, striatum, or corpus callosum, newborn SVZ neuroblasts can migrate from the SVZ to injured regions (Arvidsson et al. 2002; Goings et al. 2004; Sundholm-Peters et al. 2005). There is no agreement on whether such emigration is due to redirection of SVZ cells from the OB to the injured regions, or on increased neurogenesis, but it seems that epidermal growth factor (EGF) is the signal inducing SVZ emigration (Sundholm-Peters et al. 2005). Finally, a recent paper should be cited in this context, as it reignites the *vexata quaestio* of whether or not, in normal conditions, the neuroblasts originating from the SVZ are destined only to the OB (Breunig et al. 2007; Rakic 2002): based on thorough BrdU birthdating and retrovirus-based experiments, a significant migration of 5-HT<sub>3</sub>-positive cells is reported from postnatal SVZ into numerous forebrain regions, including the cortex, striatum, and nucleus accumbens (Inta et al. 2009).

### 11.3.1 THE ROSTRAL MIGRATORY STREAM (RMS)

The adult SVZ and the RMS are organized as an extensive network of tangentially oriented arrays, or chains, of migrating neuroblasts (Halliday and Cepko 1992; O'Rourke et al. 1995). These arrays

contain closely apposed, elongated neuroblasts connected by membrane specializations (Lois et al. 1996). The network of individual arrays is not static, but may change over time (Yang et al. 2004). Neuroblasts can move from array to array in vitro (Wichterle et al. 1997) and form new arrays in vivo (Alonso et al. 1999). In vitro studies of SVZ explants show that neuroblasts lose and reform contacts with neighbors in longitudinal arrays (Wichterle et al. 1997). Within the SVZ and the RMS, the chains of migrating cells are ensheathed by a meshwork of astrocytes originating from longitudinally oriented glial tubes that continue into the OB, wherein single neuroblasts spread radially (Jankovski and Sotelo 1996; Lois et al. 1996; Peretto et al. 1997). Chain formation is not directly linked to glial tube assembly, as it generally precedes the occurrence of complete glial ensheathment (Peretto et al. 2005).

Heterochronic and heterotopic transplantation have shown that the SVZ-OB pathway is not a "passive generic guidance" for all classes of premigratory neurons, as early postnatal (P2–13) cerebellar progenitor cells, implanted in the SVZ-OB pathway of adult mice do not migrate to the OB and acquire the phenotype of cerebellar neurons (Jankovski and Sotelo 1996).

When studied via time-lapse imaging of fluorescently labeled cells in acute brain slices, the process of cell migration in the SVZ has shown that cells move unidirectionally toward the OB with a typical leading process elongation—nuclear translocation sequence (De Marchis et al. 2001; Kakita and Goldman 1999; Suzuki and Goldman 2003).

However, a more recent paper has shown that the dynamic features of neuroblast motility in the SVZ and RMS are probably more complex than normally thought. For example, migratory morphology is not predictive of actual motility, one-third of motile neuroblasts move locally in complex exploratory patterns, and not in a fast, well-oriented way as they do for long-distance migration, and not all migrating neuroblasts are doublecortin positive (Nam et al. 2007). Tangential migration is controlled by multiple factors, including PSA-NCAM (Cremer et al. 1994; Hu et al. 1996; Ono et al. 1994; Rousselot et al. 1995; Tomasiewicz et al. 1993), extracellular matrix molecules, i.e., tenascin-C (Garcion et al. 2001; Jankovski and Sotelo 1996), and members of the ErbB and Eph family of tyrosine kinase receptors and their ligands (Anton et al. 2004; Conover et al. 2000); see Table 11.2.

#### **11.3.2** SIGNALING DRIVING THE MIGRATION

An interesting question is: How do migrating neuroblasts avoid getting lost over such a long distance and through the tangle of glial and neuronal cell bodies and processes that compose the adult brain parenchyma? It has been proposed that directional migration toward the OB is regulated by the cooperation of chemorepulsive Slit proteins expressed in the septum (Hu 1999; Hu and Rutishauser 1996) and choroid plexus (Nguyen-Ba-Charvet et al. 2004; Wu et al. 1999), and chemoattractive cues produced by the OB (Liu and Rao 2003), such as the secreted molecules netrin-1, prokineticin2, and GDNF (Murase and Horwitz 2002; Paratcha et al. 2006).

However, how can a chemorepulsive signal originating in structures separated from the SVZ by the lateral ventricle, filled with cerebrospinal fluid (CSF), and by its epithelial lining, the ependyma, orient neuroblasts migration? An elegant explanation is provided by Sawamoto et al. (2006), who show that new neurons follow the stream of CSF in the adult brain: the coordinated whiplike beating of ependymal cilia, setting in motion the CSF in a precise direction, creates a concentration gradient providing the vectorial information for guidance of the young, migrating neurons.

Although a role of the OB as a chemoattractant structure has been suggested, its involvement in proliferation and guidance of the newly born cells remains unclear. Indeed, whereas OB removal (Kirschenbaum et al. 1999) or disconnection of the olfactory peduncle (Jankovski et al. 1998) does not prevent SVZ precursors from proliferating and migrating toward the OB, a cut through the RMS (Alonso et al. 1999) or a removal of the rostral OB (Liu and Rao 2003) impedes neuroblasts migration. Thus, it has been proposed that a diffusible attractant is secreted in specific layers in the OB, including the glomerular layer, but not the GrL (Liu and Rao 2003).

After reaching the middle of the OB, the newborn cells detach from chains, migrate radially, and progress into one of the overlying cell layers, whereupon they undergo terminal differentiation. Neuroblast detachment from chains is initiated by reelin and tenascin-C, whereas radial migration depends on tenascin-R (Hack et al. 2002; Saghatelyan et al. 2004).

In the adult OB, radial glia, which guide radial migration earlier in development, are no longer present, and this poses the problem of neuroblasts guidance in this last phase of migration. A recent paper (Bovetti et al. 2007) provides a tantalizing answer: neuronal precursors would follow blood vessels, in a new form of guidance, dubbed "vasophilic." The authors provide electronmicroscopy evidence that half of the radially migrating cells associate with the vasculature in the GrL of the OB, and show in vivo time-lapse imaging demonstrating that migrating cells use blood vessels as a "scaffold" for their journey, through an interaction with the extracellular matrix and perivascular astrocyte end feet (Bovetti et al. 2007) (Table 11.3).

## TABLE 11.3 Factors Involved in Neuronal Migration

ADAM2	A disintegrin and metalloprotease 2	Contributes to RMS migration, possibly through cell–cell interactions that mediate the rapid migration of the neuroblasts to their endpoint (Murase et al. 2008)
BDNF	Brain-derived neurotrophic factor	BDNF promotes migration of SVZ neuroblasts, acting both as inducer and attractant through TrkB activation (Chiaramello et al. 2007)
Drebrin	Cytoplasmic actin-binding protein	Migrating neuroblasts in the RMS are drebrin E-immunopositive. The disappearance of drebrin E from the cell body may be a molecular switch for the cessation of migration in newly generated neuroblasts (Song et al. 2008)
EGF	Epidermal growth factor	Multiple lines of evidence suggest that EGF receptor expression correlates with cell motility neuron of progenitors in the SVZ, embryonic (Caric et al. 2001; Sun et al. 2005), and postnatal (Aguirre et al. 2005)
ErbB4	Receptor tyrosine kinase; neuregulin receptor	Expressed by the neuroblasts present in the subventricular SVZ and RMS, especially in PSA-NCAM+ (Ghashghaei et al. 2006); mice deficient in ErbB4 have altered neuroblasts chain organization and migration, and deficits in the placement and differentiation of olfactory interneurons (Anton et al. 2004; Gambarotta et al. 2004)
GABA		Migrating neuroblasts express functional GABA <sub>A</sub> receptors (Carleton et al. 2003) whose activation has a depolarizing effect (Wang et al. 2003). GABA and pentobarbital slows migratory speed via interference on intracellular Ca <sup>2+</sup> signaling (Bolteus and Bordey 2004). For a review see (Ge et al. 2007)
GDNF	Glial cell line-derived neurotrophic factor	Chemoattractant factor for neuroblast cells migrating along the RMS (Paratcha et al. 2006)
Glutamate		A mosaic expression of GluR is reported in migrating neuroblasts, including AMPA/kainate (Platel et al. 2007), NMDA (Platel et al. 2008), mGluR5 (Platel et al. 2008), and $Glu_{K5}$ -kainate, which decreases neuroblasts mobility (Platel et al. 2008)
HGF	Hepatocyte growth factor, a.k.a. scatter factor	HGF and its receptor Met protein are expressed in vivo in the OB and along the RMS; in primary in vitro cultures, HGF promotes migration of RMS neuroblasts; following point mutation of HGF, receptor neuroblast migration is reduced (Garzotto et al. 2008)
IQGAP1	IQ motif containing GTPase-activating protein 1—VEGF receptor- binding protein	Regulates VEGF-triggered neural progenitor migration in vitro (Balenci et al. 2007)

Netrin-1	Proteins involved in axon guidance	The migrating cells along the RMS express the netrin receptors neogenin and DCC; the interaction of DCC with netrin-1 contributes to the direction of migration along the RMS by regulating the formation of directed protrusions (Murase and Horwitz 2002)
Ngn2	Neurogenin 2—transcription factor of bHLH family	Ngn2 and ND1 direct differentiation of Mash1-expressing precursors into Calb+ and Calr+ neurons (Roybon et al. 2009)
NRG1- NRG3	Neuregulins, ligands of ErbB4 receptor	NRG1-NRG3 have been detected in the RMS and in adjacent regions (Anton et al. 2004)
PK2	Prokineticin 2, cysteine- rich secreted protein	Functions as a chemoattractant for SVZ-derived neuronal progenitors. Within the OB, it may also act as a detachment signal for chain-migrating progenitors arriving from the RMS (Ng et al. 2005)
PSA-NCAM	Polysialic acid (PSA), a posttranslational modification of neural cell adhesion molecule (NCAM); emophilic- binding glycoprotein	Expressed in migrating neuroblasts, is critical for tangential migration in the SVZ and RMS (Ono et al. 1994; Tomasiewicz et al. 1993); it does not affect radial migration in the OB (Hu et al. 1996)
Reelin	Product of the <i>reeler</i> gene, is an extracellular matrix glycoprotein	Promotes the change from tangential chain-migration to radial individual migration in the neuroblasts once they have reached the OB; as in the SVZ, it acts as a detachment signal, but not as a stop or guidance cue (Hack et al. 2002)
TC-C	Tenascin C—extracellular matrix glycoprotein	Expressed in the SVZ (Garwood et al. 2004) and by the astrocites forming the glial tubes in adult RMS (Jankovski and Sotelo 1996), contributes to the generation of a stem cell "niche" within the SVZ and RMS orchestrating growth factor signaling (Garcion et al. 2004)
TC-R	Tenascin R—extracellular matrix glycoprotein	Initiates both the detachment of neuroblasts from chains and their radial migration within the OB (Saghatelyan et al. 2004)
THBS-1	Thrombospondin-1— trimeric multidomain multifunctional protein	Physiological ligand for apolipoprotein E receptor 2 (ApoER2) and very low-density lipoprotein receptor (VLDLR); the loss of THBS-1 or of ApoER2/VLDLR results in severe anatomical abnormalities of the RMS and leads to a reduction of postnatal neuronal precursors entering the OB (Andrade et al. 2007; Blake et al. 2008)

## TABLE 11.3 (Continued)

## **11.4 DIFFERENTIATION**

Neurogenesis in the adult brain is confronted by two seemingly conflicting aims. On the one hand, it must maintain behavior and thus preserve the underlying circuitry, and on the other hand, it must allow circuits to adapt to environmental challenges. How are these conflicting objectives pursued in the OB?

## 11.4.1 LIFE AND DEATH OF THE NEWLY BORN CELLS

The number of cells added daily to the OB ranges from 10,000 to 30,000 (Lois and Alvarez-Buylla 1994) to 80,000 (Kaplan et al. 1985; Peterson and Peterson 2000). This would mean some 1% of the about seven million olfactory granule cell population per day in young adult rodent (Biebl et al. 2000; Kaplan et al. 1985). In contrast, neurogenesis in the SGZ of the hippocampus occurs at a considerably lower rate, about 9000 new cells per day in adult rats (Alvarez-Buylla et al. 2001), corresponding to 0.03% of the total hippocampal dentate neuronal population (Kempermann et al. 1997).

Although limited volumetric enlargement of the OB throughout lifetime has been reported in the rat (Kaplan et al. 1985), the prevailing view is that the size of the OB does not substantially change throughout life (Biebl et al. 2000; Petreanu and Alvarez-Buylla 2002; Rosselli-Austin and Altman 1979), contrary to the DG in adult rats, where neurogenesis contributes to the increase in neuronal number of granule cells (Bayer et al. 1982; Crespo et al. 1986; Imayoshi et al. 2008). The continuous generation of new neurons in the OB, in a frame of substantial stability of the total number of cells, implies that neurogenesis must be counterbalanced by an accompanying cell loss, and, in fact, programmed cell death has been shown to be a prominent regulatory feature in neurogenic regions of the OB (Biebl et al. 2000). Massive cell death has been observed during the first two months after a BrdU pulse (Winner et al. 2002). This elimination mechanism is prominent in the OB compared with the RMS and the SVZ (Belvindrah et al. 2002; Biebl et al. 2000; Petreanu and Alvarez-Buylla 2002) and may maintain a constant OB cell number by a continuous cell turnover, as was suggested during earlier development (Oppenheim 1991).

Therefore, more than the total number of newly generated cells arriving in the OB each day, what really counts is the number of newly born cells that survive and take their place in the bulbar network. In the GrL, 1 month after a BrdU pulse, this number has been estimated to range from 60,000 (Biebl et al. 2000) to 120,000 (Winner et al. 2002). In the latter case, it was shown that 50% of the newly generated neurons (i.e., about 80,000 in the GrL and 800 in the PG layer) that survived the initial period of cell death, survived for at least 19 months (Winner et al. 2002), confirming earlier work (Kaplan et al. 1985). With the use of retroviral labeling of precursors in the SVZ, it was confirmed that one-half of the labeled cells died shortly after their arrival in the OB (between 15 and 45 days after neuronal birth), and that most dying cells were mature, harboring dendritic arborization, and receiving connections (Petreanu and Alvarez-Buylla 2002). In this study, it was further shown that survival of the newly generated granule cells depends on sensory input.

A recent paper from Imayoshi et al. (2008) is a quantum leap in our knowledge of adult neurogenesis. Adopting a skillful transgenic strategy, the authors permanently label newborn neural stem cells and their progeny with a fluorescent marker, and then selectively kill new neurons at a chosen timepoint. In particular, the authors generated a mouse in which tamoxifen-inducible Cre recombinase (CreER<sup>T2</sup>) was expressed under the promoter for the neural precursor marker, nestin. By crossing their mice with a conditional LacZ reporter line, they first answer the questions of how many new stem cell-derived neurons are added to the adult brain, and whether newly generated neurons constitute a small population of neurons that are repeatedly replaced or whether they constitute a large population (Lledo et al. 2006). They find that almost the whole population of deep granule cells is replaced by new neurons over a 12-month period, whereas only half of the granule cells are replaced in the superficial layers. Other authors have also described a preferential target of adult-born granule cells to the deepest layers (ring effect; Lemasson et al. 2005; Mouret et al. 2008). This suggests that there is at least one subpopulation of persistent granule cells in the OB, which Imayoshi et al. propose might regulate the long-term memory of the smell (see below). The rate of neuronal replacement was nearly linear for the first six months, with a decrease in the pace of addition at older ages. Unfortunately, a similar quantitative analysis has not been performed for PG cells. This is probably due to the fact that PG cells constitute such a heterogeneous population that it would have required a relatively long series of double markers, so it is understandable that in a paper so rich with different approaches, this aspect has been left behind, but, nevertheless, it is a pity that this piece of information is still missing.

Next, the authors conditionally killed newborn neurons by expressing diphtheria toxin (DTA) in cells derived from nestin+ progenitors of the adult SVZ. When these new neurons were killed by DTA activation, a significant depletion of the granule cell population was observed as early as three weeks later, more evident after 12 weeks, while in controls this population remained fairly constant over time. This clearly proves that cell death is not a consequence of neuron addition, but rather is an independent process occurring even in the absence of neurogenesis. Adult neurogenesis is required to maintain a constant population of OB granule neurons in the face of normal

population turnover. Granule cells produced before the disruption of neurogenesis continued to disappear from the OB at a rate similar to that in controls, suggesting that the addition of new neurons does not elicit the death of those already present in the GrL.

## **11.4.2 ROLE OF NEUROTRANSMITTERS**

Survival and integration of newborn cells is under the control of a variety of neurotransmittermediated signaling systems, e.g., acetylcholine (ACh), glutamate, and gamma-aminobutyric acid (GABA).

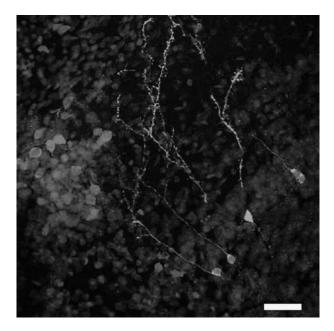
Nicotine has been shown to be detrimental to the survival of newborn granule cells in the adult OB: knockout mice lacking  $\beta$ 2 ACh receptors, the prevalent form of brain high-affinity nicotinic receptors, display nearly 50% more newborn neurons and significantly fewer apoptotic cells than control mice (Mechawar et al. 2004). Conversely, in vivo chronic nicotine exposure significantly decreases the number of newborn granule cells in wildtype but not knockout (KO) adult mice. Interestingly, KO mice, with an increased number of granule cells, have a less robust short-term olfactory memory than their wildtype counterparts (Mechawar et al. 2004).

In the hippocampus during a short critical period after neuronal birth (the third week), survival is regulated competitively by stimulation via N-methyl D-aspartate (NMDA) receptors (Tashiro et al. 2006). In the OB, there is no evidence for a similar NMDA receptor-mediated survival/death ruling, but a decrease in NMDA-mediated response in newborn PG cells seems to be important for establishing synaptic contacts with the olfactory nerve (Grubb et al. 2008).

During brain development, GABA has depolarizing activity in cerebrocortical neural precursors, controlling cell division and contributing to neuronal migration and maturation. In the adult forebrain, the SVZ and the SGZ are exposed to synaptic and nonsynaptic GABA release. Neural stem cells and neuronal progenitors express GABA receptors in SVZ. GABA effects in these cells are very similar to those found in embryonic cortical precursor cells, and therefore it is possible that this amino acid plays important roles during adult brain plasticity (reviewed in Salazar et al. 2008). Furthermore, neuronal activity accelerates neuronal differentiation and alters the mechanism of GABA synthesis in newly generated neurons (Gakhar-Koppole et al. 2008) (Figure 11.1).

## **11.4.3 GRANULE CELLS**

The majority (about 75%) of the SVZ-derived cells differentiate into GABA-containing granule cells (Betarbet et al. 1996; Carleton et al. 2003; Kato et al. 2001; Petreanu and Alvarez-Buylla 2002; Winner et al. 2002). The sequence of maturation steps marking this differentiation has been studied using a GFP-encoding retrovirus injected into the adult SVZ (Carleton et al. 2003; Petreanu and Alvarez-Buylla 2002). Before becoming a fully mature granule cell, the neuroblasts pass by a series of stages that have been well characterized electrophysiologically and morphologically (Carleton et al. 2003). In short, tangentially migrating neurons express extrasynaptic GABA(A) receptors and then  $\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors. As early as 6 days after their birth, some new neurons reach the bulb, start radial migration to their final positions, and begin to express NMDA receptors. Fourteen days after virus injection, they already display dendritic spines, suggesting they might already receive synaptic inputs. In fact, spontaneous synaptic currents have been recorded shortly after migration is complete. However, at the earlier stages of differentiation, newborn neurons remain unable to fire action potentials (Carleton et al. 2003). This delay in excitability timing, which may be finalized to protecting circuits from uncontrolled neurotransmitter release and neural network disruption, marks an interesting difference with respect to what happens during developmental neurogenesis, where spiking activity is acquired much earlier (Lledo et al. 2004). Thus, the maturation of synaptic inputs in the adult bulb does not seem to recapitulate events during embryogenesis, a difference to that which occurs in the hippocampus (Esposito et al. 2005).



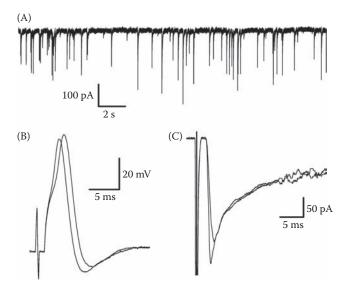
**FIGURE 11.1** (See color insert following page 206.) A newborn eGFP+ PG cell (green) around a glomerulus at P109, 3 weeks after virus injection. Calretinin-labeled (red) and tyrosine hydroxylase-labeled (blue) PG cells outline the glomerulus. Scale bar: 20 µm. (From Belluzzi, O. et al. *J. Neurosci.* 23, 10411–18, 2003. With permission.)

An interesting problem is the mechanisms regulating the way dendrites of adult-born neurons are steered at their target and compelled to establish connections with specific cell types. In principle, this could be determined either by the local environment of the neuronal circuit in the target area, or dictated by some predetermined property of the immature neurons, inherent to their particular lineage. The problem has recently been studied by Kelsh et al. (2007): using retroviral fate mapping, they studied the lamina-specific dendritic targeting of granule cells as defined by their morphology and intrinsic electrophysiological properties in neonatal and adult neurogenesis. Fate mapping revealed the existence of two separate populations of granule cell precursors, giving rise to the same neuronal type, but with two distinct patterns of dendritic targeting, innervating either a deep or superficial lamina of the external plexiform layer, where they connect to different types of principal neurons. Furthermore, using heterochronic and heterotopic transplantation and lineage tracing of neuronal precursor cells from the SVZ directly to the OB, they have elegantly revealed that the cells at an early stage of their development have a predetermined fate that is not altered by placing them in their neighboring environment. These results demonstrate that the pattern of dendritic targeting of neonatal and adult-born granule cells is a cell-autonomous property, predetermined from the moment that a neuron is born (Kelsch et al. 2007) (Figure 11.2).

## 11.4.4 PERIGLOMERULAR (PG) CELLS

Neuronal precursor cells from the SVZ differentiate into bulbar granule and PG cells with a 3:1 ratio (Kato et al. 2000; Luskin 1993; Zigova et al. 1996). Although it is certainly true that new granule cells outnumber new PG cells in the OB, the amount of the new granule cells is sometimes overestimated, as the more internal layer contains cells in the process of migration toward the glomerular layer.

Within two weeks after generation, newly generated neurons in the rat brain acquire electrophysiological properties typical of fully functional PG cells, i.e., they can fire action potentials, have well-developed, voltage-gated sodium and potassium conductances, and fully enter into

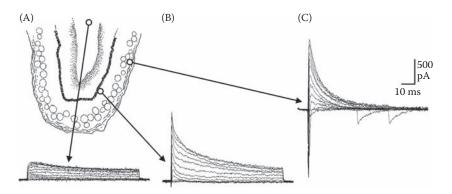


**FIGURE 11.2** Synaptic properties of newly generated PG cells. (A) Spontaneous excitatory synaptic currents in a newborn cell in the glomerular layer (P27, 12 days survival). This activity was blocked by picrotoxin, but not by kynurenate. (B) Action potentials in response to stimulation of the ON (P26, 12 days survival). (C) Synaptic currents evoked by stimulation of the ON (same cell as B). (Adapted from Belluzzi, O. et al. *J. Neurosci.* 23, 10411–18, 2003.)

synaptic relationship with other elements of the network (Belluzzi et al. 2003). This indicates that the morphological and functional differentiation of PG cells occurs rapidly and nearly concurrently within the glomerular layer.

The sequence of the development of voltage-dependent currents and synaptic connections marks the major difference between newly generated PG and granule cells. In PG cells, the maturation of voltage-dependent sodium current, and, consequently, the capacity of the newly generated cells to fire action potentials, seems to precede the appearance of synaptic contacts (Belluzzi et al. 2003), whereas in granule cells, a full development of the sodium current is observed only after the establishment of synaptic connections (Carleton et al. 2003). This difference may indicate that the pattern of functional integration of new neurons is cell-type-specific. An interesting aspect of the electrophysiological properties of newly generated cells, both PG and granule cells, is that they tend to have a sodium current significantly larger than that in controls, with a steeper conductance-voltage relationship and more negative activation voltages. This difference, as well as the higher  $g_{Na}/g_K$  ratio in new cells, may result in greater excitability to better respond to immature excitatory synaptic inputs (Belluzzi et al. 2003).

DA PG cells constitute a significant fraction of the interneurons added in adulthood to the glomerular layer (Baker et al. 2001; Betarbet et al. 1996; Winner et al. 2002; for a thorough recent review, see Cave and Baker 2009). Actually, only a minority of TH-positive cells is generated in the embryo/neonate, as the production of most of them occurs in the postnatal/adult OB (McLean and Shipley 1988; Winner et al. 2002). In the OB, DA neurons are restricted to the glomerular layer (Halász et al. 1977), but using transgenic mice expressing eGFP under the TH promoter, the presence of TH-GFP+ cells has also been detected in the mitral and external plexiform layers (Baker et al. 2001; Saino-Saito et al. 2004). Thorough studies conducted by the group of H. Baker have shown that, in some intermitral and inframitral interneurons, there is a transcription of the TH gene that is not followed by translation (Baker et al. 2001), and lead to the hypothesis that these could be adult-generated neurons committed to become DA, but not yet entirely differentiated. Accordingly, TH-GFP+ cells outside the glomerular layer exhibit functional properties (appearance of pacemaker currents, synaptic connection with the



**FIGURE 11.3** Maturation of voltage-dependent currents in newborn PG cells. Recording from cells at different stages of maturation from the positions indicated by arrows. (A) P32, 9 days survival. RMS. (B) P28, 10 days survival, just above the mitral cell layer. (C) P31, 11 days survival, glomerular layer; note in (C) the presence of synaptic currents (From Belluzzi, O. et al. *J. Neurosci.* 23, 10411–18, 2003.)

olfactory nerve, intracellular chloride concentration, and other) marking a gradient of maturity toward the DA phenotype along the mitral-glomerular axis. The establishment of a synaptic contact with the olfactory nerve seems to be the key event allowing these cells to complete their differentiation toward the DA phenotype and to reach their final destination (Pignatelli et al. 2008) (Figure 11.3).

## 11.5 FUNCTIONAL MEANING

A key question that has been associated with these studies since the initial reports that the adult brain contains stem cells that generate new neurons is whether or not adult neurogenesis is a functionally relevant process. A substantial body of work has shown that adult-born neurons can integrate into active neural circuits, but then, once they have survived, reached their target, become mature neurons, which is their function? And why is neurogenesis present only in the OB and hippocampus, and not in other areas of the brain?

The first attempts to answer these questions have been at cellular and network level, and now some enlightening answer has also been given at a higher (information processing) level.

At cellular level, differences in voltage-dependent conductance have been reported in newborn vs older PG and granule cells (Belluzzi et al. 2003; Carleton et al. 2003). However, both these reports were made in OB slices of rat, where patch-clamp recordings are very difficult after 1.5 months, so there is no evidence that these differences are maintained at older times. Other differences between adult-born and pre-existing olfactory granule neurons is in synaptic plasticity, not surprisingly much higher in newborn vs older neurons (Saghatelyan et al. 2005), and in a greater immediate early-gene (IEG) response to novel odors of adult-born granule neurons vs mature, pre-existing neurons (Magavi et al. 2005).

At network level, there is little more than plausible hypothesis (Lledo et al. 2006). Possibly, the boundaries within which effective explanations of adult neurogenesis in the OB should be searched, are defined on the one side by behavioral experiments (e.g., Rochefort et al. 2002), and on the other side by the observation that the total number of neurons in this structure remains constant in time (see above). This latter observation implies that the new neurons are not "added" to the OB, but rather replace other neurons. If one considers the OB a processing unit, then it is tempting to think of neuron swapping as an adaptive adjustment of the bulbar circuitry to better tune it to previously inexperienced external conditions. A strong argument against this possibility, lent from the neurogenesis in the hippocampus (Kempermann 2002), has been that the functional benefit from adult neurogenesis in the OB cannot be acute, because it takes several weeks to generate a functionally integrated new neuron (Ortega-Perez et al. 2007). In this view, the new connection could not benefit

the particular functional event that triggered neurogenesis, because this would be long over when the new neurons are in place. However, a recent study on the neurogenesis of DA neurons (Pignatelli et al. 2008) suggests a different way to look at the problem: new cells are continuously produced, migrate into the OB, and start to differentiate toward their final phenotype. They halt their migration in the mitral cell layer, freeze their maturation process in a preterminal state, and wait for a consensus signal that will allow them to complete their migration, and to find their place within the bulbar circuitry. This is the classical scheme also followed during embryonic development: new neurons, produced in excess, need for trophic support or synaptic input, or die (Oppenheim 1991; Oppenheim et al. 2000). In any case, this means that in any given moment there are new cells in the mitral cell layer committed to a DA fate, sending their projection into the glomerular layer and trying to establish synaptic contacts. If this does not occur, the newly generated cell will undergo apoptosis and die, and, in fact, the majority of cells generated in the SVZ are eliminated after reaching the OB (Biebl et al. 2000; Winner et al. 2002). However, if a successful synaptic contact is established, then the cell will complete its differentiation and will migrate to its final destination. It is tempting to think that, through this process, the entire circuitry of the OB can self-adapt to novel external stimuli, tailoring its wiring for optimal processing. This time gap between sensory experience and circuit modifications would be extremely small, as rewiring would require the molding of plastic elements that are *already* present in situ, and that would not need to be produced in response to the stimulus itself.

But, of course, it is mainly at behavioral level that we would like to have answers about the significance and the implications of adult neurogenesis.

The recent paper of Imayoshi et al. (2008), cited above, provides some key contributions to our understanding of the role played by adult-generated neurons in the hippocampus and, to a lesser extent, in the OB. As already mentioned, in this outstanding paper, the authors selectively killed newborn neurons by conditionally expressing DTA in cells derived from nestin+ progenitors of the adult SVZ. What, then, are the behavioral differences observed in these animals compared to wild-type mice? The selective suppression of adult-generated neurons induced severe deficits in the retention of spatial memories, attributed to the deficits in granule neuron addition to the hippocampus, but, surprisingly enough, it appeared to have little or no effect on olfactory-mediated behaviors. In a simple olfactory discrimination test, mice could still readily discriminate between odors and learn to associate specific odors with a rewarding stimulus, even six months after conditional killing of newborn neurons, when neuronal depletion in the OB was very pronounced.

The authors conclude with what could be defined a "maintenance hypothesis": in the adult OB, neurogenesis is required for the maintenance and reorganization of the entire interneuron system, but without evident roles in the acquisition of odor-associated memory. The authors, indeed, cautiously smooth their conclusion, pointing out that "more difficult tasks about odor-associated memory could depend on neurogenesis." In addition, other tasks could critically depend on the continuous rewiring of the OB circuitry ensured by adult neurogenesis, like discrimination and processing of new odors.

In any case, it must be noted that this result is at odds with other evidences suggesting that adult neurogenesis in the OB contributes to odor learning, discrimination, and adaptive behaviors in mating and pregnancy.

Discrimination learning has been reported to increase the number of newborn neurons in the adult OB by prolonging their survival. However, the simple exposure to a pair of olfactory stimuli does not alter neurogenesis, indicating that the mere activation of sensory inputs during the learning task is insufficient to alter neurogenesis (Alonso et al. 2006).

The group of Lledo has subjected NCAM-deficient mice, having severe deficits in the migration of OB neuron precursors, to experiments designed to examine the anatomical and behavioral consequences of such alteration. They found that the deficit is anatomically restricted to the GrL, and that the specific reduction in the turnover of this interneuron population resulted in an impairment of discrimination between odors. In contrast, both the detection thresholds for odors and shortterm olfactory memory were unaltered, suggesting that a critical number of bulbar granule cells is crucial only for odor discrimination, but not for general olfactory functions (Gheusi et al. 2000). The link between olfactory training and adult neurogenesis has been investigated more recently by the same group: using a discrimination learning task performed at various times after the birth of new interneurons, they found that olfactory training could increase, decrease, or have no effect on the number of surviving newly generated neurons (Mouret et al. 2008).

In adult life, the survival of newly generated neurons is critically regulated by the degree of sensory input occurring during a precise time window. Yamaguchi and Mori (2005) identified a sensitive period (14–28 days after the formation of the cells) during which sensory experience strongly influences the survival of new granule cells. It is interesting to observe that this is the time at which the new cells begin to receive glutamatergic synaptic contacts. This suggests that sensorial experience and synapse formation might be two faces of the same process, determining the survival of new granule cells during a critical period. Once rescued from death by learning, newborn neurons may remain for extended periods of time, possibly permanently.

Male pheromones stimulate neurogenesis in the adult female mice brain, and it has been shown that neurogenesis induced by dominant-male pheromones correlates with a female preference for dominant males over subordinate males, whereas blocking neurogenesis with a mitotic inhibitor eliminated this preference (Mak et al. 2007). These results suggest that regulation of adult neurogenesis by male pheromones may play an important role in reproductive strategies.

A marked increase in olfactory neurogenesis has been described during pregnancy (Shingo et al. 2003). The process is stimulated by prolactin, and inhibition of prolactin signaling results in a decrease in neurogenesis. Prolactin receptor mutant mice lack pup-induced maternal behavior, suggesting a link between new neuron production and expression of new behaviors (Shingo et al. 2003), although direct evidence that enhanced neurogenesis plays a role (rather than prolactin effecting behavioral changes through a different mechanism) still remains to be found (Temple 2003).

#### 11.6 CONCLUDING REMARKS

Many problems have to be solved before the fundamental question of the functional significance of adult neurogenesis can be fully answered.

First, we need a better description of the physiological properties of the newborn neurons. Which is, and how evolves, their excitability profile? What are the sources of the inputs these new neurons receive and which are the neurons they target? Do adult-generated neurons form circuits different from those produced during development? Of the two populations of bulbar interneurons interested in adult neurogenesis, we are starting to have good descriptions for granule cells, but not for PG cells. This is probably due to the fact that the latter are much less numerous and, additionally, there are several subtypes of PG cells (Kosaka et al. 1998), against only two, deep and superficial, of granule cells. From the more recent studies on adult neurogenesis, it is emerging that each subpopulation has its own story (Imayoshi et al. 2008), and, therefore, a systematic anatomo-functional analysis will be required to establish the role played by each subtype of newborn neurons in the existing neuronal circuits.

On a different but complementary plane, it will be important to better understand the behavioral implications of adult neurogenesis in the OB. The most obvious focus is on odor memory and discrimination, but it will not be surprising if newborn neurons contribute to these aspects in some, so far, unpredicted way.

Will neural modeling help, even guide, experimental approaches? Expectations in this sense are not missing, although, up to now, it would be difficult to cite significant contributions of computational modeling in unveiling the intimate functioning of this mysterious sense. A simple mathematical model of the OB, based on the known rules of addition of newborn neurons, has shown some capacity to organize its activity in order to maximize the difference between its responses, self-adapting to changing environmental conditions (Cecchi et al. 2001). Possibly, more sophisticated models in the near future will provide interesting working hypothesis.

The challenge posed by the sense of smell is still open, and more than ever enticing, with many of the relevant mechanisms involved in odor recognition still escaping our full understanding. Adult neurogenesis is only the last of a long series of surprises bequeathed by this sense, and promises that the efforts in unveiling its secrets will be all but boring.

## ACKNOWLEDGMENT

This work was supported by a grant from MIUR (PRIN 2007).

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## 12 Active Sensing in Olfaction

Matt Wachowiak

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## 12.1 INTRODUCTION

A fundamental feature of sensory systems is that the animal can actively control the interaction between a stimulus and the sensory neurons detecting it. This active control is important because it allows an animal to sample regions of interest in space, to regulate stimulus intensity in order to maintain optimal receptor function, to extract features of interest from a complex stimulus, and to protect sensory neurons from damage due to excess exposure to strong or (in the case of chemoreception) toxic stimuli. Active sensation is especially prominent in olfaction; in vertebrates, for example, odorants cannot be detected without the movement of air or water into the nasal cavity, and vertebrates and invertebrates alike have impressively complex behavioral repertoires built around the process of sampling odorants. This chapter will focus on the importance of active sensing to olfactory system function. A key point is that active sensing is important not only in shaping how sensory neurons respond to a stimulus, but also in determining how incoming sensory information is processed at higher levels, modulated by behavioral state, and, ultimately, perceived by the animal. For example, active odorant sampling constrains the temporal structure of sensory input to the nervous system, a feature that probably has important consequences for how the postsynaptic networks that process olfactory information are designed and function. At the same time, sampling behavior is tightly linked to behavioral state, so that "top-down," state-dependent modulation of sensory processing probably goes hand-in-hand with "bottom-up" changes in the nature of sensory input. Finally, in order to correctly process incoming information, sensory processing must be coordinated with the motor systems involved in stimulus sampling. This chapter will review how active sensing shapes olfactory system function at each of these levels. Because of the wide-ranging nature of the subject, treatment of individual topics is not exhaustive; the reader is referred to a number of excellent, more focused reviews at the end of the chapter. In addition, relevant chapters in this volume are cited where possible to minimize overlap of content.

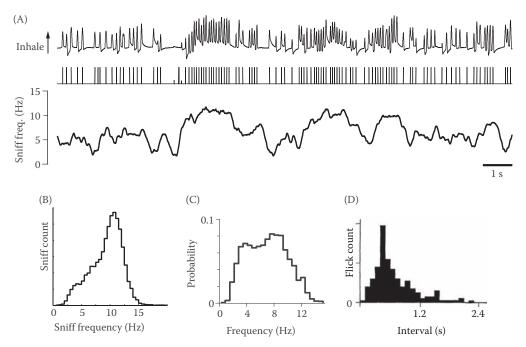
#### 12.2 ODORANT SAMPLING BEHAVIOR

Most terrestrial vertebrates sample odorants by drawing air into the nasal cavity and over the olfactory epithelium (OE). Odorants are usually sampled intermittently, either during the course of resting respiration or by the voluntary inhalation of air in the context of odor-guided behavior; the latter phenomenon is typically termed sniffing. Analogs of sniffing occur across the animal kingdom, with groups as diverse as crustaceans (Snow 1973; Koehl et al. 2001), fish (Nevitt 1991), semiaquatic mammals (Catania 2006), and insects (Suzuki 1975; Lent 2004) showing active, intermittent odorant sampling; in each case, sampling involves movement of the air or fluid containing the stimulus by the animal, or movement of the olfactory organ itself (for a review, see Dethier [1987]). For example, lobsters "flick" their olfactory organs (antennules) when sampling odorant-laden water (Schmitt and Ache 1979; Koehl et al. 2001), while the air-breathing shrew samples odorants underwater with an "inverted" sniff, in which air is partially exhaled onto a substrate and then reinhaled (Catania 2006). A common feature of all of these behaviors is that they are actively controlled by the animal and modulated depending on the properties of the stimulus itself (e.g., odorant concentration or hedonic value) (Youngentob et al. 1987; Bensafi et al. 2003; Johnson et al. 2003), the particular sensorimotor task being performed (e.g., detection vs scent-tracking) (Thesen et al. 1993), and behavioral context (e.g., exploration vs reward-based conditioning) (Clarke 1971; Lent and Kwon 2004; Kepecs et al. 2007). The persistence of this behavior in different species and ecological settings as well as its strong modulation during odor-guided behaviors (Figure 12.1), suggests that active, intermittent sampling of odorant is fundamentally important to olfaction (Dethier 1987).

Odorant sampling behavior (i.e., sniffing) has been most comprehensively studied in mammals—particularly in rodents and humans. In rodents in particular, sniffing is precisely coordinated with other motor systems and is highly dynamic, with many parameters of a sniff varying on a cycle-by-cycle basis (Figure 12.1A) (Welker 1964; Macrides et al. 1982; Youngentob et al. 1987). The parameter of sniffing that has received the most attention and which changes most clearly in rodents is frequency: respiratory frequency increases from "resting" rates (near 2 Hz in larger rodents such as rats and hamsters; 3–5 Hz in mice) to rates ranging from 6 to 12 Hz when investigating novel odor sources or sampling odorants during operant tasks (Welker 1964; Macrides et al. 1982; Youngentob et al. 1987; Uchida and Mainen 2003; Verhagen et al. 2007; Kepecs et al. 2007; Wesson et al. 2008). Rats also alter other parameters of sniffing during odor-guided behavior, including amplitude, inhalation–exhalation waveform, and duration (Youngentob et al. 1987; Youngentob 2005). In humans, inhalation amplitude, duration, and number of sniffs are modulated during odorant sampling (Laing 1982, 1985; Sobel et al. 2000a). Changes in these parameters alter the instantaneous rate and total volume of airflow over the OE during a sniff (Youngentob et al. 1987; Sobel et al. 2000a).

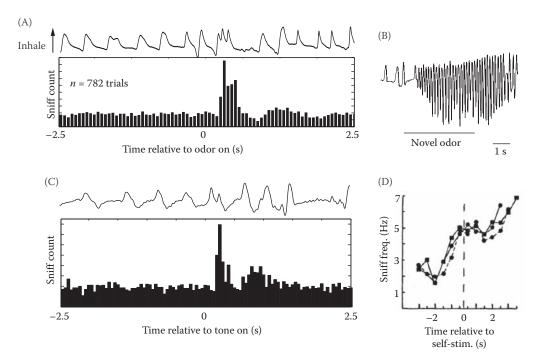
While complex and dynamic, sniffing behavior is precisely controlled by the animal and can be surprisingly stereotyped (Figure 12.2A and B). For example, when sampling odorant from a delivery port in an operant two-odor discrimination task, rats show a brief bout of 6–10 Hz sniffing that is precisely timed to just precede odorant delivery, and a slightly higher-frequency sniff bout (10–12 Hz) just prior to receiving a water reward; each of these bouts is repeated with a temporal jitter of only a few hundred milliseconds across hundreds of trials (Kepecs et al. 2007; Wesson et al. 2009). Humans also show stereotyped and task-dependent sniffing patterns (Laing 1982), and are also capable of rapidly modulating sniffing in response to sensory input (Johnson et al. 2003).

Thus, the pattern of sniffing expressed during a particular behavior can be thought of as a strategy for odorant sampling; these strategies are task and context specific and can be expected to vary between species as well as across individuals within a species. For example, rats increase sniff frequency and amplitude as odorant concentration approaches threshold values when performing an odor-detection task (Youngentob et al. 1987), but not when performing an odor-discrimination task (Wesson et al. 2009). Similarly, mice show a stereotyped bout of high-frequency sniffing when performing a two-odor discrimination task involving a nose poke into a sampling port, but not when



**FIGURE 12.1** Odorant sampling behavior in different animal species. (A): Sniffing behavior in a freely moving mouse. Shown are raw recordings of intranasal pressure measured from the dorsal recess (top trace), a raster of individual sniff onsets (middle), and a moving average of sniff frequency over time. Sniffing varies over a wide range and is rapidly modulated. (B): Histogram of sniff frequency in mice exploring a novel environment. Sniff frequency varies from ~1 to ~15 Hz, with a peak at 10 Hz during exploratory behavior. (C): Histogram of sniff frequency in a rat performing a two-odor discrimination task. Sniff frequency varies across a similar range as in mice. (D): Intervals of antennular flicking in the hermit crab *P. alaskensis.* Flicking frequencies (1/interval) range from ~0.5 to 10 Hz, with a peak at ~2 Hz. ([A] and [B]: From Wesson, D.W. et al. *PLoS Biol.*, 6, e82, 2008a. [C]: From Kepecs, A., Uchida, N., and Mainen, Z.F., *J. Neurophysiol.*, 98, 205–13, 2007. [D]: From Snow, P.J., *J Exp Biol.*, 58, 745–65, 1973.)

performing the same discrimination task involving sampling odorant in cups of sand (Wesson et al. 2008b). Both rodents and humans show individual differences in sniffing behavior when sampling odorants (Wesson et al. 2009; Laing 1983). One might expect that odorant sampling strategies are optimized to the particular context (and individual) in which they are expressed. Indeed, measurement of sniff parameters in humans performing odor threshold and intensity tasks indicates that those expressed naturally by each subject are near-optimal for performance in the task; increasing the number of sniffs or varying sniff interval or magnitude leads to no improvement in performance over that during natural sniffing (Laing 1983, 1985). A striking example of context-specific sampling strategies in a more ethologically natural setting is seen in bird-hunting dogs: when tracking the scent of prey on the ground, dogs sniff at up to 4-6 Hz, but when tracking the same scent in the air, the animal will raise its head and run forward, forcing a continuous stream of air into the nose for up to 40 s (Thesen et al. 1993; Steen et al. 1996). The presumed advantage of this latter strategy is to enable a continuous sampling of odorant while the dog is moving at high speed and to decouple sampling from respiration during a time of heavy load on the respiratory system. Similarly, rodents exhibit prolonged bouts of sniffing at 4–8 Hz when sampling a novel odorant (Figure 12.2C), but show only brief or no increases in sniff frequency when sampling a familiar odorant for the purposes of odor discrimination (Welker 1964; Macrides 1975; Kepecs et al. 2007; Verhagen et al. 2007; Wesson et al. 2009). Brief sniffing (in fact, only a single sniff) appears to provide sufficient contact with the stimulus to enable odor identification (Goldberg and Moulton 1987; Uchida and Mainen 2003; Wesson et al. 2008a), while prolonged high-frequency sniffing is probably useful in



**FIGURE 12.2** Sniffing behavior in different olfactory and nonolfactory contexts. (A): Histogram of sniffing relative to time of odorant presentation in a head-fixed rat performing a lick/no-lick two-choice odor discrimination. This rat displays a brief bout of high-frequency sniffing, which occurs just after odorant onset. (B): Sniffing in a head-fixed rat in the same paradigm as in (A), but after presentation of a novel odorant. The novel odorant elicits a prolonged bout of high-frequency (approximately 6 Hz) sniffing. (C): Histogram of sniffing relative to time of tone presentation in a head-fixed rat; same paradigm as in (A), but measured in a rat performing an auditory discrimination task. The rat also displays a brief bout of high-frequency sniffing just after tone onset. (D): Increase in sniff frequency evoked by self-administered electrical stimulation of the lateral hypothalamus. Sniffing increases just prior to the animal beginning self-stimulation and persists throughout the stimulation period. ([A]: From Wesson, D.W., Verhagen, J.V., and Wachowiak, M., *J Neurophysiol.*, 101, 1089–1102, 2009. [B]: From Verhagen, J.V. et al. *Nat. Neurosci.*, 10, 631–39, 2007. [C]: From Wesson, D.W., Verhagen, J.V., and Wachowiak, M., *J Neurophysiol.*, 101, 1089–1102, 2009. [D]: From Clarke, S., *Physiol. Behav.*, 7, 695–99, 1971.)

gathering additional information about the location, spatial distribution, or dynamics of an odorant that is novel (Verhagen et al. 2007).

Finally, in analyzing sampling behavior and its role in olfaction, it is important to remember that, in the awake animal, sniffing (or its analog) is typically expressed as part of a larger behavioral repertoire that may include head movements, whisking (in rodents), licking, and locomotion (Welker 1964; Komisaruk 1970; Bramble and Carrier 1983). The tight coordination of sniffing with other behaviors can confound the interpretation of the role that sampling behavior plays in the process of olfaction. For example, sniff frequency may increase in animals that are actively engaged with their environment due simply to increased demand on the respiratory system. In addition, it is difficult to isolate sniffing behavior from the expression of other behaviors associated with active sensory sampling. For example, mice and rats increase sniff frequency in response to unexpected stimuli of any modality (Welker 1964; Macrides 1975; Harrison 1979), and increase sniff frequency when approaching and inserting their nose into a port—even when performing nonolfactory tasks (Figure 12.2D) (Wesson et al. 2008b, 2009). Mice and rats also increase respiratory frequency prior to receiving a reward and when otherwise engaged in motivated behavior (Figure 12.2E) (Clarke 1971; Clarke and Trowill 1971; Kepecs et al. 2007; Wesson et al. 2008b). Thus, in practice it is difficult to find criteria that define "sniffing" as a behavior solely associated

with odorant sampling and distinct from respiration. As we will see later in this chapter, such distinctions are also difficult when considering the role of sampling behavior in shaping the neural processing of olfactory information: the same changes in behavioral state associated with sniffing can modulate olfactory processing through neural mechanisms, even at the lowest synaptic levels of the olfactory pathway.

### 12.3 EFFECT OF SAMPLING BEHAVIOR ON RECEPTOR NEURON ACTIVATION

Odorant sampling behavior plays a fundamental role in the neural coding and processing of odor information because it controls the access of odorant molecules to the sensory neurons themselves. In terrestrial vertebrates, for example, inhalation of air is *required* for olfactory receptor neurons (ORNs) to detect an odorant. More importantly, sampling behavior can directly shape receptor activation in two ways. First, intermittent odorant sampling imposes a strong temporal structure on the dynamics of ORN activation. Second, changes in sampling behavior can rapidly modulate the strength and, potentially, the patterns of ORN activation by changing the nature of airflow through the nasal cavity. Each of these effects is important for encoding odor information and for processing olfactory sensory input downstream.

# 12.3.1 SAMPLING BEHAVIOR SHAPES THE TEMPORAL STRUCTURE OF RECEPTOR NEURON ACTIVATION

The temporal dynamics of ORN activation depend strongly on sampling behavior. In rodents, ORNs are not activated when odorant is simply blown at the nose; the animal must inhale for odorant to reach the (Wesson et al. 2008a). Once inhalation begins, ORN activation occurs relatively quickly: calcium imaging from the presynaptic terminals of ORNs reveals that odorant-evoked action potentials first reach the olfactory bulb (OB) 80–160 ms after the start of inhalation (Figure 12.3A and B) (Wesson et al. 2008a; Carey et al. 2009). This time is surprisingly short given published estimates of 150–600 ms for transduction times in ORNs in vitro (Firestein et al. 1990; Ma et al. 1999). Activation timing relative to inhalation is also precise, with response onset latencies varying by only approximately 50 ms from sniff to sniff during low-frequency sniffing (Carey and Wachowiak, pers. comm.). Inhalation-driven responses are transient: both presynaptic calcium imaging and electroolfactogram recordings from awake, freely breathing rats suggest that each inhalation of odorant evokes a burst of ORN input to an OB glomerulus, lasting 100–200 ms (Chaput and Chalansonnet 1997; Verhagen et al. 2007; Carey et al. 2009). Whether the transient nature of the ORN response is due to rapid clearance of odorant from the receptor site or rapid adaptation of ORNs (Reisert and Matthews 2001) is unclear.

The initial response to odorant involves a progressive recruitment of activation of the population of ORNs that converge onto a single glomerulus over a time-window of at least 80–100 ms, rather than a synchronous activation (Figure 12.3B) (Carey et al. 2009). This value is similar to the rise-time of odorant-evoked excitatory postsynaptic potentials (EPSPs) in mitral/tufted (M/T) cells of anesthetized, freely breathing rats (Cang and Isaacson 2003; Margrie and Schaefer 2003), consistent with the idea that M/T cells integrate ORN inputs over the time-window of a single sniff. Surprisingly, behavioral measurements of odor perception times in awake rats, performed simultaneous with imaging of ORN inputs to the OB, indicate that at least some forms of odor identification occur *before* this initial response onset phase is even finished (Wesson et al. 2008a); studies of olfactory reaction times in rodents and rabbits are consistent with this conclusion (Karpov 1980; Uchida and Mainen 2003; Abraham et al. 2004; Rinberg et al. 2006b). Thus, the initial onset phase of the inhalation-evoked burst of ORN activity is likely to be particularly important for olfactory processing. How the relatively slow, asynchronous recruitment of ORN inputs to a glomerulus shapes this processing and contributes to odor coding has yet to be explored, either in experimental preparations or via modeling of neural processing in the OB.

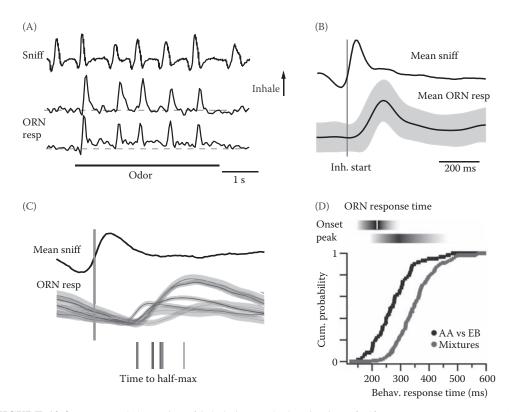


FIGURE 12.3 Temporal dynamics of inhalation-evoked activation of olfactory receptor neurons. (A): Intranasal pressure ("sniff," top) and receptor neuron response traces ("Glom 1," "Glom 2") imaged from two glomeruli in the olfactory bulb of an awake, head-fixed rat. Response traces are estimates of receptor neuron firing rates based on presynaptic calcium imaging. (B): Mean receptor neuron response pattern following inhalation of odorant. Gray shading indicates variance (standard deviation) around the mean. This trace represents the mean response averaged across many (>5500) odorants and glomeruli. (C): Mean receptor neurons response evoked by the same odorant in different glomeruli. Traces are "sniff-triggered averages" of evoked presynaptic calcium signals. Sensory input to different glomeruli occurs with different latencies and rise-times. Vertical lines below trace indicate time to half-max for each glomerulus. (D): Dynamics of receptor neuron response after one sniff match the dynamics of odor perception. Gray scale bars at top indicate range of onset latencies ("onset") and range of times-to-peak ("peak") of presynaptic calcium signals imaged in awake, head-fixed rats. Plots show behavioral response times in mice performing a go/no-go two-odor discrimination. The shortest response times (for easy discriminations) match the earliest presynaptic onset latencies, while the longest response times (for difficult mixture discriminations) match the longest times-to-peak of the presynaptic signals. ([A]: From Verhagen, J.V. et al. Nat. Neurosci., 10, 631-39, 2007. [B, C]: From Carey, R.M. et al. J Neurophysiol., 101, 1073-88, 2009. [D]: From Abraham, N.M. et al. Neuron, 44, 865-76, 2004.)

A number of studies have suggested that ORNs may be activated by respiration alone, independent of odorant stimulation. This issue remains controversial, but is important in that inputs driven by inhalation alone would provide direct signals to the OB about the timing of sampling behavior. Many studies have reported respiratory patterning of postsynaptic activity in the OB in the absence of odorant (Adrian 1942; Macrides and Chorover 1972; Chaput et al. 1992; Rinberg et al. 2006a); others have found no such patterning or have observed patterned responses in some OB neurons but not others (Walsh 1956; Sobel and Tank 1993). A recent study of mouse ORNs recorded in vitro found that up to 60% of all ORNs responded to pressure pulses that were estimated to approximate pressure transients generated during sniffing, that these responses were absent in ORNs from mice missing components of the second messenger pathway that mediates odorant responses, and that respiration-linked field potentials in the OB were disrupted in these mice (Grosmaitre et al. 2007). A different study using presynaptic calcium imaging from ORNs in awake rats found that inhalation alone evoked detectable ORN input to a similar fraction (at least 50%) of glomeruli in the dorsal OB; the magnitude of inputs to most of these glomeruli was small, although inhalation evoked large-magnitude inputs to a few glomeruli (Carey et al. 2009). More work is needed to resolve whether the in vivo results reflect a mechanosensitive capability in some ORNs or, instead, reflect responses to odorants emitted by the animal or other environmental changes related to respiration (e.g., temperature or carbon dioxide level).

In addition to being shaped by the respiratory cycle, the temporal dynamics of ORN activation are intrinsically variable: temporal response parameters, such as latency, rise-time, and burst duration, vary for ORN inputs to different glomeruli, for the same ORN inputs activated by different odorants, and, to some degree, by concentration (Figure 12.3C). Diverse response dynamics are seen both in anesthetized and awake mice and rats, with the particular temporal pattern of ORN activation occurring reliably across multiple respiratory cycles and consistently for homologous glomeruli in different animals (Spors et al. 2006; Carey et al. 2009). Thus, these dynamics do not appear to be an artifact of the calcium-imaging method used to detect them. Instead, intrinsic temporal response patterns probably reflect odorant-specific differences in the kinetics of odorant access to the ORNs as well as ligand/receptor interactions. The significance of this temporal diversity is that patterns of sensory input to OB glomeruli evolve over time in an odorant-specific manner, and so may play a role in coding odor information. In awake rats, the time-window over which patterns of glomerular input evolve (the time from the earliest activated inputs to the peak of the latest activated inputs) is approximately 250 ms (Carey et al. 2009). This window roughly matches the amount by which discrimination time increases when mice and rats are asked to perform more difficult odor discriminations (Figure 12.3D) (Abraham et al. 2004; Rinberg et al. 2006b). Thus, the intrinsic variability in the dynamics of inhalation-evoked ORN inputs to the OB may set an upper limit on the time-window for integration of odor information in the behaving animal.

Increasing the frequency of respiration and odorant sampling—e.g., during exploration of a novel environment or active odor investigation-dramatically alters the temporal structure of ORN activity patterns. The main effect of high-frequency sniffing is to reduce the degree to which ORN activation is linked to the respiratory rhythm. As respiration frequency increases (from 1 to 2 Hz at rest to above 4 Hz during active sniffing in rats), the temporal coherence between the respiratory cycle and ORN activation dynamics diminishes; for many ORN populations (as defined by their convergence onto different OB glomeruli), ORN responses become tonic, with no clear modulation by the respiratory cycle (Verhagen et al. 2007; Carey et al. 2009). This effect is at least partly a result of reduced modulation of odorant levels in the OE: simulations of bulk airflow through the rat nasal cavity and odorant sorption into the OE indicate that odorant is not fully cleared during high-frequency sniffing (Zhao and Jiang 2008). Measurements of pressure transients resulting from active sampling of odorant also suggest that high-frequency sniff bouts involve a net influx of air into the nose (Youngentob et al. 1987). Thus, during high-frequency sniffing, exposure of ORNs to odorant changes from being transient to being continuous (though still modulated in absolute level), resulting in a tendency of ORNs to respond more tonically. This qualitative change in the temporal structure of ORN activation probably has significant consequences for postsynaptic processing of odor information; these are discussed in more detail in Section 12.4.

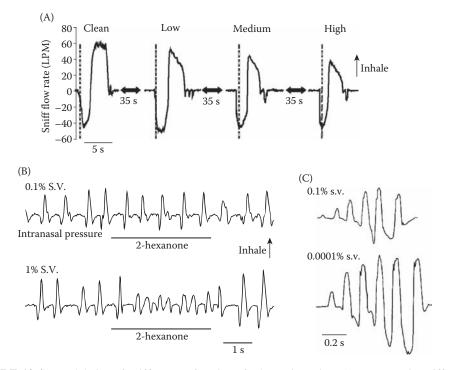
#### 12.3.2 SAMPLING BEHAVIOR CAN SHAPE PATTERNS OF RECEPTOR NEURON ACTIVATION

Odorant sampling behavior also has the potential to modulate the strength and relative pattern of activation of ORNs. This modulation can be mediated by changes in the total volume (i.e., mass) of inhaled odorant, changes in the flow rate, and changes in sniff frequency, all of which affect the dynamics of odorant exchange in the nasal cavity. An animal may actively modulate each of

these parameters, and adjustment of each parameter may optimize ORN responses for different odor-guided tasks.

Modulation of sniff volume has long been hypothesized to play a role in maintaining odor quality perception across different intensities. Animals encounter odorants over a wide range of concentrations, and must maintain at least some degree of constancy in quality perception across this range. While human psychophysical studies suggest that odor quality perception can vary with large changes in odorant concentration, this perception is relatively invariant over one to two orders of magnitude (Laing et al. 2003). However, many studies characterizing odor representations in the OB of anesthetized rodents have found that the pattern of activated ORNs or their corresponding glomeruli can change dramatically over a concentration range of 1 log unit or less (Rubin and Katz 1999; Meister and Bonhoeffer 2001; Wachowiak and Cohen 2001; Bozza et al. 2004). This result is expected, given that increasing odorant concentration activates increasing numbers of ORN types (Malnic et al. 1999). If the combination of activated ORNs or OB glomeruli encodes odor quality, then how is quality perception maintained across concentration? One possibility is that adjustments in sampling behavior may compensate for intensity changes by sampling more or less odorant per inhalation. This process would be analogous to the pupillary reflex in the eye.

The strongest evidence for such an effect comes from work in humans. Subjects performing odorant intensity estimates will suppress the strength of a single sniff at high-odorant intensities, resulting in a reduction in the total volume of inhaled odorant (Figure 12.4A) (Laing 1982; Warren



**FIGURE 12.4** Modulation of sniffing as a function of odorant intensity. (A): Decrease in sniff magnitude (peak inhalation and exhalation flow rate) as odorant intensity increases from low to high (progressing left to right) in humans performing an odor intensity judgment. (B): Decrease in inhalation amplitude in head-fixed rats as odorant intensity increases from low (top, 0.1% s.v.) to moderately high (bottom, 1% s.v.). Note that the first inhalation (before the odorant is detected) shows no change. (C): Increase in sniff magnitude and frequency as odorant intensity decreases from suprathreshold (top trace) to near-threshold (bottom trace) in rats performing an odorant detection task. ([A]: From Johnson, B.N., Mainland, J.D., and Sobel, N., *J. Neurophysiol.*, 90, 1084–94, 2003. [B]: Data provided by Verhagen, J. and Wachowiak, M. et al. *J Neurophysiol.*, 90, 1084–95, 2003. [C]: From Youngentob, S.L. et al. *Physiol Behav.*, 41, 59–69, 1987.)

et al. 1992; Johnson et al. 2003). This modulation of sniffing is surprisingly fast—as fast as 160 ms—leading to the hypothesis that subcortical pathways may mediate this response (Johnson et al. 2003; Mainland and Sobel 2006). One caveat in interpreting these data is that some odorants used in these studies can activate nasal trigeminal afferents at high concentrations, triggering a reflexive change in sniffing as a result of nasal irritation (Warren et al. 1994; Benacka and Tomori 1995); nonetheless, modulation of sniffing by high concentrations of "pure" olfactory stimuli can occur in as little as 260 ms (Johnson et al. 2003). Similar to this, our laboratory has observed that rats suppress inhalation amplitude when exposed to moderately high concentrations of certain odorants (Figure 12.4B) (Wesson and Wachowiak, pers. obs.). In at least some behavioral paradigms, animals will attempt to sample more odorant as concentration decreases. For example, rats performing an odor-detection task show higher-amplitude and higher-frequency sniffs as concentration nears their perceptual threshold (Figure 12.4C) (Youngentob et al. 1987). Similarly, human subjects performing a detection task and forced to sniff through only one nostril will increase sniff duration when sniffing through the low flow-rate nostril as compared to the high-flow-rate nostril (nasal patency is asymmetric in macrosmatic mammals, and alternates from side to side every few hours [Principato and Ozenberger 1970; Bojsen-Moller and Fahrenkrug 1971]), a behavior consistent with compensatory sniffing at near-threshold intensities (Sobel et al. 2000a). In summary, there is considerable evidence that animals actively adjust sniff parameters as a function of odorant intensity to facilitate concentration-invariant odor perception. However, it is important to recognize that, to date, no study has actually examined how-or even whether-such changes in sniff volume impact the neural representation of odorants in the periphery or in the central nervous system (CNS) in a manner consistent with this idea.

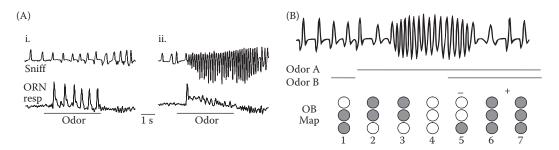
Another longstanding hypothesis is that modulating sniffing behavior can cause changes in flow rate that shape ORN response patterns by altering how odorant distributes across the OE (Adrian 1950; Mozell 1964). This idea—which we will call the sorption hypothesis—arises from the fact that odorant molecules must pass from an airborne vapor phase to an aqueous phase in the OE in order to contact ORNs. The nasal cavity of most vertebrates—and mammals in particular—is anatomically complex and forms a narrow airspace lined with epithelium onto which odorant molecules absorb as they flow through the cavity (Keyhani et al. 1995; Craven et al. 2007; Yang et al. 2007). This arrangement causes a "chromatographic effect," in which odorants are preferentially absorbed in different locations depending on their solubilities in the mucus and their flow rate (Mozell and Jagodowicz 1973; Yang et al. 2007). The topography of odorant receptor expression across the OE correlates with the areas of maximal sorption for the receptors' respective ligands, suggesting that receptors are optimally localized to take advantage of the chromatographic effect (Scott et al. 2000; Schoenfeld and Cleland 2006). Because the strength, duration, and frequency of respiration can change dramatically during odor-guided behavior and because these parameters affect the rate and total volume of airflow into and out of the nasal cavity, sampling behavior has the potential to alter odorant sorption and, as a consequence, patterns of ORN activation (Mozell et al. 1987; Youngentob et al. 1987). This phenomenon is described in more detail elsewhere in this volume (Chapter 13) and in several excellent reviews (Schoenfeld and Cleland 2005, 2006; Scott, 2006).

Indeed, many studies have confirmed that flow rate impacts the spatial distribution of odorant sorption across the OE, and that this, in turn, shapes both spatial and temporal patterns of ORN activity (Kent et al. 1996; Scott 2006). Physiological studies and detailed modeling of airflow and sorption in the nasal cavity have generated specific predictions about how flow rate should shape activity in the intact animal (Mozell et al. 1987; Hahn et al. 1994; Zhao et al. 2006; Yang et al. 2007). The most directly testable is the following: at low flow rates, strongly sorbed odorants will be largely removed from the airstream as they pass through the initial parts of the epithelium, resulting in fewer odorant molecules available to activate more posterior odorant receptors. At higher flow rates, more molecules of strongly sorbed odorant reach the posterior epithelium and so evoke responses that increase with increasing flow rate. In contrast, weakly sorbed odorants absorb slowly

onto the epithelium and so tend to remain in the airstream. For these compounds, a bolus of odorant passing through the nasal cavity would deposit fewer odorant molecules onto the epithelium at high-flow rates than at low, since the bolus would pass through the nasal cavity with insufficient time for complete sorption to occur. Thus, responses to a strongly sorbed odorant should increase as flow rate increases, while responses to a weakly sorbed odorant should decrease (Hahn et al. 1994). Such effects have, in fact, been measured at the level of the OE, by adjusting nasal flow while measuring electroolfactogram responses or imaging membrane potential across the epithelial surface (Kent et al. 1996; Scott-Johnson et al. 2000; Scott et al. 2006; Mozell et al. 1991). One intriguing piece of behavioral evidence supporting the idea that sorption effects can shape odor quality perception comes from a study in humans, in which subjects judged the relative magnitude of each component of a binary mixture while sniffing through only one nostril. When sniffing through the lower flow-rate nostril, subjects judged the weakly sorbed odorant to be more intense, and judged the strongly sorbed odorant to be more intense when sniffing through the higher flow-rate nostril (Sobel et al. 1999), consistent with the sorption hypothesis. Together, these studies confirm that the parameters of respiration have the potential to alter primary odor representations, and raise the possibility that animals might alter sampling behavior in a way that generates an optimal odor representation (and perception) for a particular olfactory task (Mainland and Sobel 2006; Schoenfeld and Cleland 2006).

These studies have some important limitations, however. First, both the odorant uptake modeling and physiological studies have, for the most part, used steady-state flow rates, not the transient changes in airflow that occur during natural respiration and active sniffing. Whether the sorption effects seen with steady-state flows manifest differently during natural respiration has not, to our knowledge, been tested either computationally or physiologically. Second, actual flow rates in the nasal cavity are difficult to measure directly: total inspiratory and expiratory flow has been measured reliably in behaving rats and mice (Youngentob et al. 1987; Youngentob 2005), but the proportion of that flow passing over the olfactory region and its subcompartments has only been estimated by modeling. It also remains unclear whether the different sampling strategies expressed in behaving animals alter airflow sufficiently to alter ORN responses according to predictions. Finally, it remains unclear what impact these changes at the level of the OE will have on odor representations at the level of the OB. Thus, while chromatographic effects have the *potential* to shape patterns of ORN activity as a function of sampling behavior, the sorption hypothesis has yet to be tested under conditions of natural odorant sampling and at the level of neural patterns of activity in the CNS.

A third way in which sampling behavior can alter ORN response patterns-including at the level of the OB—is through changes in sniff frequency. As already described, animals strongly modulate sniff frequency and typically engage in bouts of high-frequency sniffing when investigating novel stimuli or exploring an environment (Welker 1964; Macrides 1975), and these increases shape the temporal structure of ORN responses (Carey et al. 2009). Sniff frequency also shapes the magnitude of ORN responses, although in unexpected ways. An intuitive prediction is that increases in sniff frequency lead to increased ORN responses-and perhaps recruitment of activation of new ORN populations—due to an increased influx of odorant per unit time. This prediction has been tested using presynaptic calcium imaging from ORN terminals in the dorsal OB of awake rats, which sampled the same odorant during low frequency (1–2 Hz) respiration or during high frequency (4–8 Hz) sniffing (Verhagen et al. 2007). Surprisingly, sampling an odorant at high frequency only weakly enhanced the initial response to the odorant, and did not recruit activation of new ORN populations. More importantly, sustained high-frequency sniffing of odorant had the opposite effect, with a strong attenuation in the magnitude of ORN responses (Figure 12.5A). This frequency-dependent attenuation is rapidly reversible, with ORN response magnitudes recovering within 1 s after sniff frequency returns to low levels. The effect is also not driven by changes in behavioral state, as it can be replicated during artificial "playback" of high-frequency sniffing patterns in anesthetized rats (Verhagen et al. 2007). A likely cellular mechanism mediating the frequency-dependent attenuation of ORN inputs to the OB is simple adaptation of ORN spiking. Rat ORNs in vivo respond to step



**FIGURE 12.5** Adaptive filtering of sensory inputs controlled by sniffing. (A): Rapid attenuation of receptor neuron activation during sustained high-frequency sniffing of an odorant. Top traces show sniffing in a head-fixed rat, lower traces show receptor neuron responses, estimated from presynaptic calcium signals as already described. Responses are reliable across inhalations when sampled at low frequency (left), but show rapid attenuation during high-frequency sniffing of the same odorant. (B): Schematic illustrating the effects of high-frequency sniffing on odor representations in a changing odor landscape. Top trace, representative sniffing pattern, including a bout of high-frequency sniffing. Lower graphics ("OB map") represent patterns of activation of glomeruli in the olfactory bulb at different times (1–7) during sampling of odorants A and B. When sampled in isolation, A and B have overlapping glomerular representations (1–2). High-frequency sniffing of odor A causes an attenuation of the response map (3–4); when odor B is encountered against the background of odor A, only those glomeruli that differ from those activated by odor A are activated (5). A return to low-frequency sniffing removes the attenuation and the representation changes to resemble the sum of the two single-odor maps (6–7). Thus, changes in the odor landscape are represented in a subtractive or additive mode depending on sniff frequency. ([A]: From Verhagen, J.V. et al. *Nat Neurosci.*, 10, 631–39, 2007.)

odorant pulses with brief (<100 ms) action potential bursts (Duchamp-Viret et al. 2000), and receptor currents in isolated mouse ORNs show ~80% adaptation within 2 s of continuous odorant exposure (Reisert and Matthews 2001). At low respiration rates, ORNs can recover from adaptation in the interval between successive inhalations, but higher sniff frequencies allow less time for recovery between cycles and probably also include a tonic component in which odorant is continuously present in the nasal cavity (Youngentob et al. 1987; Uchida and Mainen 2003; Zhao and Jiang 2008).

What is the functional significance of this phenomenon? Importantly, frequency-dependent attenuation of ORN responses is specific to those glomeruli receiving odorant-evoked input, leaving other glomeruli free to respond to other odorants encountered during a sniff bout. As a result, this attenuation constitutes an "adaptive filter" of sensory input to the OB, in which ORNs activated by odorants present at the beginning of exploratory sniffing (i.e., "background" odorants) are selectively suppressed in the representation of subsequent sampled odorants (Figure 12.5B) (Verhagen et al. 2007). In contrast, during low-frequency sampling, odorants encountered against a background are encoded as the sum of the background and "foreground" response maps. This filtering can enhance the contrast between odorants having overlapping molecular features (or mixtures with shared components). A second important function of frequency-dependent attenuation is to increase the salience of temporally dynamic or spatially localized odorants relative to broadly distributed background odorants. These properties seem optimally suited for scanning the environment for changes in odor composition or concentration, and may explain why high-frequency sniffing is induced by any novel stimulus or during general exploratory behavior (Welker 1964; Vanderwolf and Szechtman 1987). Humans also modulate sniff duration during active odor sampling (Laing 1982, 1983; Sobel et al. 2000a); in this case, prolonged odorant inhalation may also attenuate receptor inputs via adaptation, enhancing the ability to detect changing olfactory stimuli in a single long sniff.

A key feature of all of the above phenomena is their direct dependence on odorant sampling parameters. Thus, most animals have a surprising degree of control over the way in which a complex and dynamic odor landscape is represented at the level of sensory input to the CNS. These low-order effects are likely to be magnified by processing in higher-order networks, and further modulated by top-down processes driven by changes in behavioral state, as discussed in more detail below.

# 12.4 ACTIVE SAMPLING AND THE POSTSYNAPTIC PROCESSING OF OLFACTORY INFORMATION

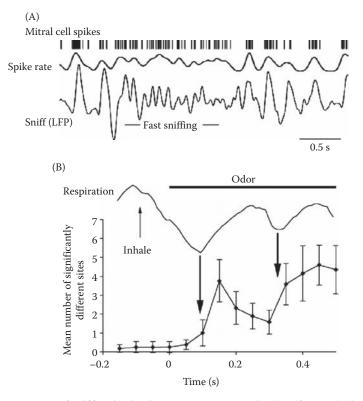
It seems obvious to expect that the processing of olfactory information in the CNS will depend on the temporal structure and magnitude of inputs from ORNs, and so will be strongly shaped by sampling behavior. Indeed, temporally patterned activity relative to respiration is a key feature of most models of information coding and processing in the mammalian OB. For example, many experimental and computational studies have suggested that olfactory information is encoded in the timing of mitral cell spiking relative to the respiratory cycle or to the OB theta rhythm (Macrides and Chorover 1972; Chaput 1986; Hopfield 1995; Margrie and Schaefer 2003; Buonviso et al. 2006; Schaefer and Margrie 2007); details of these studies are described elsewhere in this volume (Chapter 13). However, most previous studies have explored odor coding in the OB and beyond during sampling regimes that are both low frequency (1–3 Hz) and highly regular. Neither of these features apply to active odor sampling (see Figure 12.1). Thus, how the OB network processes odor information during odor-guided behaviors, and, specifically, how changes in sampling behavior associated with particular sampling strategies shape synaptic processing, remains unclear.

#### 12.4.1 EFFECTS OF SAMPLING BEHAVIOR ON OLFACTORY BULB (OB) PROCESSING

Active sampling and olfactory processing have been examined mostly in the context of sniff frequency, and explored most heavily in the OB. A great deal of evidence suggests that during low-frequency respiration (1–2 Hz), the OB network enhances the temporal patterning that is present at the level of ORN inputs and increases the temporal precision and synchrony of firing of the principal output neurons of the OB. For example, in OB slice preparations, delivering patterned olfactory nerve stimulation at frequencies that roughly mimic resting respiration alters responses of postsynaptic neurons: external tufted (ET) cells become entrained to this input and synchronized with each other, M/T cell responses are amplified and synchronized, and gamma-frequency oscillations in M/T cell membrane potential emerge (Schoppa and Westbrook 2001; Hayar et al. 2004; Schoppa 2006). Modeling studies support these data: for example, a compartmental model of mitral cell firing properties predicts that ORN inputs that arrive at the OB in bursts (as they do during resting respiration) will cause an increase in the temporal patterning and spike timing precision of mitral cells (David et al. 2007). These phenomena arise from multiple mechanisms in the glomerular and subglomerular layers, the details of which are described elsewhere (see Chapter 13).

Very few studies have investigated how sniffing in the high-frequency (4–12 Hz) range alters postsynaptic response properties. Recordings from M/T cells in awake, freely moving rats show that M/T firing largely decouples from respiration at sniff frequencies above 4 Hz and adopts a more tonic pattern (Figure 12.6A) (Bhalla and Bower 1997; Kay and Laurent 1999). Decoupling was originally interpreted as reflecting state-dependent modulation of M/T cell responses by centrifugal inputs. However, this effect could simply be due to the reduction in temporal patterning of ORN inputs that occurs during high-frequency sniffing (Verhagen et al. 2007; Carey et al. 2009). A more recent study (Bathellier et al. 2008) artificially controlled sampling frequency in anesthetized mice, and reported that temporal patterning was maintained at high frequencies, but that response magnitudes were attenuated. This attenuation is consistent with the fact that ORN inputs become attenuated during high-frequency sniffing in awake rats (Verhagen et al. 2007).

Since the functional properties of the postsynaptic OB network are dynamic, it is also likely that changes in the frequency and temporal structure of ORN input lead to *qualitative* changes in the way that this network processes olfactory information during active sampling. For example, the synapse between ORNs and second-order neurons is subject to strong activity-dependent depression due to feedback presynaptic inhibition and vesicle depletion, and postsynaptic  $\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors show rapid and strong desensitization (Murphy et al. 2004; McGann et al. 2005; Wachowiak et al. 2005). Thus, tonic or high-frequency ORN inputs



**FIGURE 12.6** Importance of sniffing in shaping response patterns in the olfactory bulb and the piriform cortex. (A): In an awake, freely moving rat, a mitral/tufted cell shows firing rate increases locked to sniffing at low frequencies, but loses temporal patterning during high-frequency sniffing. Top, spike raster; middle trace, smoothed spike rate; lower trace, local field potential (LFP) recorded from the OB, indicating sniffing behavior. (B): Dynamics of population activity patterns of neurons in the piriform cortex relative to inhalation. Neuronal response patterns become distinct from one another (i.e., show odorant-specific activity) within 200 ms after inhalation of odorant, and reach peak divergence within the first cycle. These dynamics are similar to those seen at the level of receptor input to the OB (see Figure 12.3). ([A]: From Kay, L.M. and Laurent, G., *Nat Neurosci.*, 2, 1003–9, 1999; Rennaker, R.L. et al. *J. Neurosci.* 27, 1534–42, 2007. [B]: From Rennaker, R.L. et al. *J. Neurosci.* 27, 1534–42, 2007.

would suppress the overall strength of afferent synaptic drive onto M/T cells. Second, recurrent inhibition between mitral and granule cells enhances the temporal precision of M/T cell firing when excitatory inputs to M/T cells themselves are temporally patterned (Balu et al. 2004; Schoppa 2006), but modeling and experimental studies suggest that this precision is reduced or lost when inputs are temporally dispersed or occur tonically (Balu et al. 2004; David et al. 2007). Recurrent inhibition between mitral and granule cells may also be stronger at higher sniff frequencies (Young and Wilson 1999). These findings predict a reduction in M/T cell temporal patterning during high-frequency sniffing. A third prediction is that increasing sniff frequency leads to an increase in the strength of inhibition both within and between glomeruli due to an enhanced synchrony and strength of firing of cells. ET cells drive both feed-forward and feedback inhibition within the glomerular layer and become more synchronous and increasingly entrained to rhythmic ORN inputs as sniff frequency increases (Hayar et al. 2004). Thus, at high-sniff frequencies, ET cell-driven inhibition is predicted to generate an increasingly sharp time-window over which M/T cells may integrate ORN inputs (Wachowiak and Shipley 2006). As this temporal window narrows, only those M/T cells innervating glomeruli receiving the fastest-onset input may be activated. Increased ET cell activation at higher sniff frequencies may also increase the strength of *interglomerular* inhibition, because ET

cells can also drive inhibition in neighboring glomeruli via short-axon interneurons (Aungst et al. 2003). The net result of these effects would be to sharpen overall M/T cell response patterns.

Thus, the changes in sniff frequency associated with active sensing have the potential to qualitatively alter how olfactory information is represented at the level of output from the OB. However, exactly how sniffing behavior changes odor representations at this level remains largely hypothetical. These predictions need to be tested using a combination of in vivo recordings (ideally, in awake animals), OB slice recordings using naturalistic input patterns mimicking different sampling behaviors, and modeling studies.

#### 12.4.2 ACTIVE SAMPLING EFFECTS BEYOND THE OLFACTORY BULB (OB)

Even fewer data exist on the effect of sampling behavior on odor representations beyond the OB. As with the OB, most studies characterizing response properties of neurons in higher olfactory centers (primarily in the piriform cortex [PC]) have been performed in anesthetized animals breathing at low and regular rates. Nonetheless, these studies support the idea that temporally dynamic ORN inputs are integrated on a cycle-by-cycle basis during sniffing. For example, odorant-specific patterns of activation across small populations of neurons in the PC develop over the first 100–200 ms after inhalation (Nemitz and Goldberg 1983; Rennaker et al. 2007), a time-course similar to that of ORN input patterns (Figure 12.6B). PC neurons also show distinct temporal patterning relative to the respiratory cycle (Wilson 1998; Litaudon et al. 2003; Rennaker et al. 2007).

One effect of sampling behavior that may be important at the level of the cortex is laterality in odor sampling. Olfactory inputs remain unilateral at the level of the OB, but can cross sides at the level of the anterior olfactory nucleus via the anterior commissure. Interestingly, neurons in the PC show different degrees of laterality, with most neurons driven by ipsilateral inputs, but some driven by bilateral or strictly contralateral inputs (Wilson 1997). Thus, neurons in the cortex may be involved in comparing the strength of odorant input through the two nares and also in integrating inputs across the two nostrils. Both of these computations may be useful in tracking odors or possibly detecting gradients of odor intensity: for example, humans can follow an odor trail laid on a solid substrate more successfully when using two nostrils rather than one (Porter et al. 2007), and rats can be trained to detect differences in odor intensity and timing of arrival of odorant across the two nostrils (Rajan et al. 2006).

The PC also shows relatively rapid habituation to prolonged odorant stimulation: in the rat, PC pyramidal cells habituate within 30–40 s (Wilson 2000) (see also Chapter 14), and in humans, fMRI signals reflecting neural activity in the PC show habituation with a nearly identical time course (Sobel et al. 2000b). Habituation in the PC is odorant-specific, and so may facilitate the separation of background and foreground odorants (Kadohisa and Wilson 2006; Linster et al. 2007). This phenomenon is similar to the adaptive filtering that occurs at the level of sensory input to the OB during high-frequency sniffing (Verhagen et al. 2007), although in the cortex this adaptation occurs passively and during baseline respiration. It will be interesting to explore how cortical habituation functions during active sniffing and as sampling changes from moment to moment in the behaving animal.

## 12.5 TOP-DOWN MODULATION OF OLFACTORY PROCESSING DURING ACTIVE SENSING

While sampling behavior can shape ORN and postsynaptic responses via the "bottom-up" mechanisms described above, olfactory information processing is also subject to top-down modulation. In humans, attention to an olfactory task modulates activity in primary olfactory cortical areas (Zelano et al. 2005). In the OB, response properties of M/T cells in rats and mice performing odorguided tasks can change rapidly depending on stimulus context (Karpov 1980; Kay and Laurent 1999), stimulus valence (Doucette and Restrepo 2008), whether an odorant is being actively or passively sampled (Fuentes et al. 2008), and other behavioral states related to active sensation, such as attention, arousal, and motivation (Karpov 1980; Tsuno et al. 2008). Since sniffing is tightly linked to such behavioral states, active bottom-up and top-down mechanisms are likely to be closely coordinated. Other forms of modulation less clearly associated with active sensing—such as during the sleep/wake cycle and after associative conditioning—are discussed elsewhere in the book (Chapters 14 and 15).

Active, top-down modulation of olfactory processing is likely to be mediated by multiple centrifugal systems, and probably occurs at all levels of the central olfactory pathway. Three neurotransmitter systems that have been strongly implicated in top-down modulation related to active sensing are acetylcholine, serotonin, and norepinephrine. Centrifugal cholinergic neurons originate from the horizontal limb of the diagonal band of Broca (HDB) and project to both the OB and the PC (Macrides et al. 1981; Luskin and Price 1982; Carson 1984). In other sensory modalities, the cholinergic system plays a role in attentional modulation of sensory processing, where it is generally thought to enhance processing by amplifying the signal-to-noise ratio of attended sensory responses relative to ongoing background activity (Sarter et al. 2005; Hasselmo and Giocomo 2006). Cholinergic modulation has been strongly implicated in shaping odor coding and perception in a manner consistent with this idea. For example, systemic administration of the cholinesterase inhibitor, physostigmine, enhances rats' ability to perform difficult odor discrimination tasks (Doty et al. 1999; Linster 2002; Mandairon 2006), and selective lesion of cholinergic neurons in the HDB increases rats' generalization between similar odorants (Linster et al. 2001). At least some of these modulatory effects occur as early as the OB; local application of cholinesterase inhibitors sharpens odorant specificity of M/T cells and increases spontaneous discrimination of similar odorants, while nicotinic or muscarinic antagonists in the OB decrease behavioral discrimination (Mandairon et al. 2006; Chaudhury et al. 2009). These results predict that enhanced activation of the HDB inputs to the OB during active odor sampling or during perceptual learning increase the ability of the early olfactory system to form distinct representations for similar odors (Fletcher and Wilson 2002; Linster and Cleland 2002). At the circuit level, these effects are probably mediated—at least in part—by modulation of inhibition between mitral and granule cells (Elaagouby et al. 1991; Tsuno et al. 2008), although the strong cholinergic innervation of the glomerular layer suggests that these inputs also modulate signal transfer from sensory inputs to the mitral cell primary dendrite.

Serotonergic afferents to the OB originate in the dorsal and median raphe and most heavily innervate the glomerular layer, with less robust inputs to subglomerular layers (McLean and Shipley 1987; Gomez et al. 2005). Depletion of these afferents results in deficits in olfactory learning (McLean et al. 1993), although this effect may result from interactions with the noradrenergic inputs to the OB (Price et al. 1998), whose role in associative conditioning to odors is described elsewhere in this book (Chapter 14). The circuit mechanisms underlying serotonergic modulation remain unclear, although recent evidence suggests that a major effect of serotonin is to increase the excitability of GABAergic periglomerular interneurons (Hardy et al. 2005; Petzold et al. 2009). One effect of such modulation in vivo is to suppress odorant-evoked transmitter release from ORNs via GABABmediated presynaptic inhibition (Aroniadou-Anderjaska et al. 1999; McGann et al. 2005; Petzold et al. 2009). Thus, in addition to other potential roles, serotonergic afferents from the raphe can regulate the gain of sensory input to OB glomeruli. Interestingly, serotonergic neurons in the raphe have an important link to active sensing in the somatosensory system: neurons in the caudal raphe target premotor neurons in the facial nucleus and activate a central pattern generator that drives whisking during somatosensory exploration (Hattox et al. 2003; Cramer et al. 2007). Whisking and sniffing are each rhythmic, dynamically controlled behaviors that are typically expressed together as part of the same behavioral sequence and reflect a state of active investigation of the environment (Welker 1964; Komisaruk 1970). One intriguing possibility, then, is that activation of serotonergic neurons in the raphe drives active whisking and simultaneously modulates the processing of olfactory inputs to the OB.

The OB also receives strong innervation from noradrenergic inputs originating in the locus coeruleus, with fibers primarily targeting subglomerular layers (Shipley et al. 1985; McLean et al. 1989). This system—like the serotonergic and cholinergic systems—has been implicated in modulation of sensory processing as a function of arousal or attention. In the awake animal, locus coeruleus neurons are strongly activated by novel stimuli, and are thought to be important in driving exploratory behavior and in optimizing the coding and processing of sensory information (Aston-Jones and Bloom 1981; Sara et al. 1994, 1995; Hurley et al. 2004; Aston-Jones and Cohen 2005). Noradrenergic modulation in the OB affects odor-discrimination behaviors in rats in a manner similar to that of cholinergic modulation, enhancing the discrimination between similar odorants (Doucette et al. 2007; Mandairon et al. 2008). The projection patterns of noradrenergic inputs suggest that they modulate inhibition between mitral and granule cells, although the circuit mechanisms by which this modulation occurs and the contribution by different adrenergic receptor subtypes appears complex (Mandairon et al. 2008). Noradrenergic inputs from locus coeruleus also probably modulate the responses of neurons in the PC (Bouret and Sara 2002).

Finally, there are strong centrifugal projections from the PC and other higher-order olfactory centers—including the anterior olfactory nucleus, the entorhinal cortex, the amygdala, and the ventral hippocampus (Shipley and Adamek 1984; van Groen and Wyss 1990; McLean and Shipley 1992). Centrifugal afferents from the PC and anterior olfactory nucleus are presumed to be glutamatergic; those from the PC target the granule cell layer (Shipley and Adamek 1984), while those from the anterior olfactory nucleus target multiple OB layers (Brunjes et al. 2005). The role of any of these inputs in olfactory processing remains unclear, although the feedback from the PC to the OB has been hypothesized to mediate rapid, online modulation of OB output during odor-guided behavior. One interesting model predicts that odorants are identified with increasing precision with each successive cycle of feedback between the OB and the cortex, with each cycle driven by a sniff (Ambros-Ingerson et al. 1990). Centrifugal cortical inputs may also be important in shaping the temporal response properties of M/T cells during sniffing, either by providing a signal phase-locked to the sniff cycle that affects M/T cell spike timing (Margrie and Schaefer 2003; Kay 2005), or by triggering a switch from phasic to tonic firing modes when the animal switches from low- to high-frequency sniffing (Bhalla and Bower 1997; Kay and Sherman 2006).

#### 12.6 SENSORIMOTOR INTEGRATION IN OLFACTION

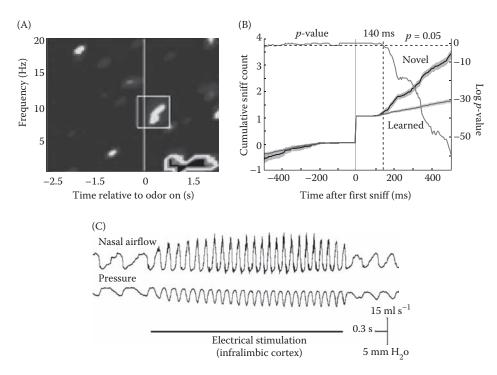
A hallmark of active sensing in other sensory systems is their close association with the motor pathways controlling stimulus sampling. For example, in the visual and auditory systems, a saccade to actively sample a region of visual space increases the responsiveness of tectal and cortical neurons with receptive fields in the same region (Goldberg and Wurtz 1972; Winkowski and Knudsen 2006); this spatial attentional modulation is controlled by neurons in the gaze control centers of the brain, which send reafferent signals to sensory areas (Moore et al. 2003; Winkowski and Knudsen 2006). Likewise, in the rodent somatosensory system, responses of neurons in the barrel cortex (the primary cortical area for sensory input from the whiskers) are rapidly suppressed during active whisking (Ferezou et al. 2006; Hentschke et al. 2006), and monosynaptic connections exist between the primary sensory and motor cortices corresponding to the same whisker, providing a substrate for tightly controlled sensorimotor integration (Ferezou et al. 2007).

These examples from other sensory systems lead to the prediction that signals reflecting the motor drive to sniffing might shape sensory processing at early stages of the olfactory pathway. The strongest evidence in support of this idea comes from the fact that neurons in the OB show modulation in firing rate in phase with respiration, even in the absence of odorant (Adrian 1942; Walsh 1956; Macrides and Chorover 1972; Chaput and Holley 1979). Interpretation of these data is confounded by the likelihood that ORN inputs themselves are activated by respiration, as described above. However, several studies have found that respiration alone—when decoupled from nasal airflow in tracheotomized animals—can shape temporal response patterns in OB mitral cells, although inhalation-driven

inputs appear to be a stronger determinant of response timing (Ravel et al. 1987; Ravel and Pager 1990; Sobel and Tank 1993). This issue remains controversial; one possibility is that centrifugal inputs reflecting the sniff cycle are activated only during active sniffing and not during passive respiration.

Nonetheless, temporal coupling between the dynamics of neural activity in the olfactory pathway and rhythmic odor sampling is one of the most robust features of the olfactory system (Adrian 1942; Macrides and Chorover 1972; Macrides 1975). This coupling probably plays an important role in mediating odor-guided behavior. For example, sniffing transiently synchronizes with the theta rhythm in the hippocampus during investigative sniffing (Macrides et al. 1982), and the magnitude of this coupling is correlated with performance on a two-odor discrimination task (Figure 12.7A) (Kay 2005). One explanation for this relationship is that sniff timing is adjusted to synchronize with hippocampal theta during active odor sensing (Macrides 1975; Macrides et al. 1982), rather than the theta rhythm being driven by sniff-related reafferent signals (Kay 2005).

It is also clear that the olfactory sensory inputs can strongly influence the motor systems controlling sniffing. First, as described above, olfactory stimuli can modulate sniffing behavior extremely rapidly—within approximately 200 ms after beginning an inhalation (Figure 12.7B) and in as little as 50–100 ms after sensory input arrives at the OB (Johnson et al. 2003; Wesson et al. 2008a). In fact, the spontaneous modulation of sniffing behavior in response to a novel odorant is faster than the conditioned response to a rewarded odorant (Wesson et al. 2008a). Analysis of the timing of



**FIGURE 12.7** Sensorimotor integration underlying sniffing behavior. (A): Coherence between field potential recordings from the olfactory bulb and dorsal hippocampus during performance of a two-odor discrimination task. Lighter colors indicate higher coherence. There is a transient increase in coherence in the theta band at the time of expected odor sampling. (B): Rats show rapid changes in sniffing behavior in response to a novel odorant. Shaded plots show cumulative sniff count over time during presentations of novel vs learned odorants. Upper plot shows *p*-value of the difference between novel and learned trials. Sniffing behavior diverges significantly at 140 ms. (C): Electrical stimulation of infralimbic cortex in an anesthetized rat elicits respiratory changes that closely resemble exploratory sniffing (compare with Figure 12.2B). ([A]: From Kay, L.M., *PNAS.*, 102, 3863–68, 2005. [B]: From Wesson, D.W. et al. *Chem Senses.*, 33, 581–96, 2008b. [C]: From Aleksandrov, V., Invanova, T.G., and Aleksandrov, N.P., J. Physiol. Pharmacol., 58, 17–23, 2007.]

individual sniffs relative to odorant presentation in rats performing odor-guided tasks indicates that animals can (and do) modulate their sniffing behavior on a cycle-by-cycle basis (Kepecs et al. 2007; Wesson et al. 2008a, 2009); this is an impressive feat, as cycle-by-cycle control of sniffing in the frequency range of 4–10 Hz suggests a sensorimotor control loop requiring well under 200 ms.

The neural pathway underlying this sensorimotor loop is unclear. In humans, suggests the speed of this response that it may be mediated by a subcortical pathway, at least in humans (Johnson et al. 2003). An important component of this pathway may be the cerebellum, which is activated during sniffing, may receive olfactory input from the PC, and is involved in optimizing motor output for sensory acquisition in other modalities (Sobel et al. 1998; Johnson et al. 2003; Mainland and Sobel 2006). The hippocampus has also been proposed to play a role in controlling sniffing behavior in response to olfactory inputs (Vanderwolf, 1992, 2001); this hypothesis arises from findings that gamma-frequency (30–80 Hz) activity in the dentate gyrus occurs during active sniffing, but not in response to other sensory inputs (Vanderwolf 2001), and that theta-frequency activity (2–10 Hz) synchronizes with sniffing during active odor sampling (Macrides 1975; Macrides et al. 1982). The pathway from the hippocampus to the motor centers controlling sniffing has not been elucidated. Finally, it is still possible that cortical centers play an important role in olfactory sensorimotor integration. Interestingly, electrical stimulation of the insular cortex and infralimbic cortex in anesthetized rats alters respiration; stimulation of the infralimbic cortex, in particular, elicits increases in respiration frequency that are remarkably similar to exploratory sniffing (Figure 12.7B) (Aleksandrov et al. 2007). This cortical pathway may be relatively short: there are direct connections between the OB and the anterior insular cortex and, possibly, the infralimbic cortex (Shipley and Adamek 1984); both cortices, in turn, send projections to the parabrachial nucleus, which participates in respiratory rhythm generation (Moga et al. 1990).

Finally, there is evidence from work in humans that information about the motor control of sniffing can significantly influence odor perception. First, in human subjects in which odorant is "presented" via the bloodstream by intravenous injection, sniffing appears to "gate" perception of an odor (Mainland and Sobel 2006). Second, the amount of effort expended in a sniff affects perceived odor intensity. Increases or decreases in flow rate caused by manipulating airflow resistance during constant sniffing lead to changes in perceived intensity in the direction predicted by the effect of flowrate on ORN responses (Hahn et al. 1994); however, the same changes in flow rate caused by voluntary changes in sniff magnitude (i.e., inhalation pressure) generally do not lead to perceived intensity changes (Teghtsoonian et al. 1978; Teghtsoonian and Teghtsoonian 1984; Youngentob et al. 1986; Hornung et al. 1997). These results suggest that motor information about sniffing is rapidly integrated with incoming sensory information, and that the motor component is an essential part of the construction of an odor percept (Mainland and Sobel 2006).

#### 12.7 SUMMARY

As in other sensory systems, the sampling of olfactory stimuli is tightly controlled by the animal, with important consequences for information coding, processing, and perception. Indeed, considering olfaction as a system in which stimulus sampling, behavioral state, motor system function, and information processing strategies are closely coordinated is fundamental to understanding olfaction in the behaving animal. This chapter touched on how active sensing is important and integrated at each of these levels. For a more detailed review of the relationship between odor sampling and nervous system function at a particular level, the reader is referred to several excellent reviews (Schoenfeld and Cleland 2005, 2006; Buonviso et al. 2006; Mainland and Sobel 2006; Scott 2006; Wachowiak and Shipley 2006).

#### ACKNOWLEDGMENTS

I would like to thank the past and present members of the Wachowiak laboratory, in particular D. Wesson, J. Verhagen, and R. Carey, for contributing to the viewpoints expressed here and for performing the critical experiments described from our laboratory. I would also like to thank H.

Eichenbaum, M. Shipley, D. Katz, A. Fontanini, A. Yamaguchi, and K. Zhao for valuable discussions on topics presented here. The laboratory has been supported by grants from the National Institutes of Health (NIDCD) and from Boston University.

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# 13 Temporal Coding in Olfaction

Brice Bathellier, Olivier Gschwend, and Alan Carleton

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# 13.1 INTRODUCTION

Knowledge about the molecular organization principles of the sense of smell in different species has greatly improved in the last decade (Bargmann 2006; Mombaerts 2004a, 2004b; Rodriguez 2007). It is now well established that in many species, odorant molecules are detected by large families of G-protein-coupled receptors (Buck and Axel 1991), whose molecular sequence and structure may vary across species and phyla, but that essentially implement the same function (Bargmann 2006). Interestingly, the insect olfactory receptors display a unique and unconventional membrane topology in comparison to the mammalian receptors, questioning the existence of a coupling with G-proteins (Benton et al. 2006; Vosshall and Stocker 2007). Nevertheless, understanding how odorant information generated by these large arrays of receptors is interpreted

by the brain to produce a great variety of behaviors will be the challenge of the next decade. A few questions, which may appear basic with regard to the complexity of the entire olfactory system, are still not answered. Among these, how olfactory information is encoded in brain networks down-stream to receptors, remains poorly understood. In recent years, there have been strong debates on this question and it seems that the answer is not as simple as recording from the neurons of these networks. The ambition of this chapter is not to provide a definitive answer, but to present the most relevant results on this question and put them in perspective, helping the reader to appreciate where the field stands in terms of olfactory coding. Since a certain similarity in the olfactory system organization has been observed across species (Kay and Stopfer 2006), we will endeavor to compare between different animal models. Our focus will primarily be on temporal coding, as temporal dynamics, in our opinion, are currently the main aspect of neuronal activity in the olfactory system that is difficult to integrate in a convincing and unanimously recognized theory of olfactory coding.

In most, if not all species, the olfactory system has a first stage downstream to receptor neurons, where sensory axons converge in a receptor-specific fashion onto projection neurons (PNs) dendritic tuft, forming segregated anatomical structures called glomeruli. These downstream structures are found in the main olfactory bulb (OB) in vertebrates or the antennal lobe (AL) in insects, which are considered as functionally analogous in many studies (Kay and Stopfer 2006). So, the first step into the system goes with a clean separation of the different information channels. The information then goes forward via the output neurons of the OB (mitral and tufted cells) or the AL (PNs) to several downstream areas. At this level, the system starts to diverge into the brain. However, in vertebrates as in insects, one of these areas receives more massive projections and is considered to have a more central role than others. This would be the piriform cortex in mammals or the mushroom body (MB) in insects. Most of the present debates about olfactory coding focus on the first stage (bulb or AL), wondering how the circuitry of these networks might transform the spatially segregated sensory input. An increasing number of studies also started to address the question of coding in the main target areas of the OB and AL. Both levels will be reviewed in this chapter.

The first basis of olfactory coding corresponds to the fact that the large receptor repertoire is expressed in a very controlled manner, as one sensory neuron usually expresses only a single olfactory receptor (Bargmann 2006). In most cases, receptors are sensitive to many chemical compounds and have overlapping receptive fields (Firestein 2001; Hallem and Carlson 2006). This suggests that odor identity is represented by complex combinations of receptor activations rather than by the activity of a specific receptor. However, there are also some cases of highly selective receptors. For example, the two receptors, Gr21a and Gr63a, expressed in the *Drosophila* olfactory epithelium, uniquely respond to  $CO_2$  (Jones 2007; Kwon et al. 2007) and drive innate avoidance behavior (Suh et al. 2004, 2007). In mice, the existence of a "specialist glomerulus" narrowly tuned to a compound present in urine was reported (Lin et al. 2005), though the receptor may still be activated by other nontested chemicals. The concentration seems to be a factor modulating the sensitivity range. With higher concentration of an odorant, more receptors become activated (Hallem and Carlson 2006). Hence, the initial olfactory code is sparser at low than at high concentrations.

This discrepancy between specialists and generalists olfactory receptors is the subject of another debate. Some researchers defend a view of the olfactory system where activity of an olfactory receptor is transmitted further in a dedicated "labeled line" pathway, which receives only very limited or no interactions with other pathways. This idea is supported by the observation that, in some animals such as mammals or *Drosophila*, second order neurons (mitral cells, PNs) receive sensory inputs from a single receptor type. Others oppose that at each level of the olfactory system, olfactory information is largely distributed in the network, in part due to lateral interactions between neurons. This idea is supported by the existence of lateral, multisynaptic connections between second order neurons both in the OB (Shepherd 1972) and the

AL (Olsen et al. 2007; Olsen and Wilson 2008). The answer to this debate is probably that both schemes coexist. It was recently shown in *Drosophila* that among two different narrowly tuned olfactory receptors, one was connected to a very specific downstream neuron of the AL, suggesting a "labeled line" pathway, and the other was connected to a broadly tuned neuron, suggesting contribution to a distributed code (Schlief and Wilson 2007). This outcome may be only partially surprising if one considers that the olfactory system might be involved both in simple, stereotyped, and eventually innate behaviors, as well as in more complex behaviors learned throughout life. "Labeled line" circuits might be present to fulfill simple but important functions, such as carbon dioxide detection in insects (Jones 2007). An innate avoidance circuit, only relying on the dorsal glomeruli of the OB, has recently been described in mice (Kobayakawa et al. 2007). If these glomeruli are genetically inactivated, mice can still learn avoidance to the innately repulsive odors (i.e., can still somehow recognize them), but do not show the innate avoidance behavior anymore.

All this must be kept in mind when addressing the question of odor coding. The OB and the AL are probably less functionally homogenous than one might think, and have several different targets. Each target network does not read out the same code as other targets, meaning that several "codes" may coexist, either in different neurons or in the same neurons. Hence, the coding schemes that we will describe in the following paragraphs have to be interpreted as potentially specific to a particular function and one does not necessarily exclude the other.

This chapter has three parts. After reviewing the temporal constraints on olfactory perception (Section 13.2), we will describe the different types of temporal dynamics observed in the olfactory system (Section 13.3). Finally, we will expose and discuss the current hypotheses on how these dynamics might contribute to the odor code (Section 13.4).

#### 13.2 OLFACTORY PERCEPTION IN THE TIME DOMAIN

All perceptions occur in time, and time is essential for proper perception and discrimination. Therefore, it is obvious that temporal constraints will most likely influence the selection of a code for sensory information transfer. The purpose of this section is to introduce the concept of temporal coding, to characterize the temporal constraints, and figure how they might determine the choice of a neural code used for odor perception.

#### 13.2.1 TEMPORAL CODING: DEFINITION AND CONTROVERSY

It is a rather difficult task to define what is meant by temporal coding, as different authors may have implicitly used different definitions. Here, we suggest using the definition proposed by Dayan and Abbott (2001). For these authors, a temporal code is a code based on temporal relationships in the neural response. Temporal relationships can either be timing of the response and, more generally, temporal sequence of the response relative to some clock signal (e.g., stimulus onset, oscillation), or timing of neurons with respect to each other in a population (e.g., synchrony, neuronal activation sequences). It is important to note that a firing rate computed in a specific time window does not make use of any temporal relationship in the responses, and is, therefore, not a temporal code. However, a code based on a series of rate measures in time is a temporal code, as it uses the temporal sequence of neural activations. In consequence, Dayan and Abbott argued that temporal coding and rate coding should not be opposed, as they might be nested in the same scheme. Only the special case (although used in many studies on sensory coding) of a firing rate measure in a single time window can plainly be opposed to temporal coding.

Unfortunately, for some authors, the existence of temporally patterned neural responses does not by itself indicate a temporal code, as neural signals can be read in many ways that do not necessarily include temporal relationships. However, Dayan and Abbott (2001) proposed

a method to assess if temporal coding is plausible or not. Temporal coding is only possible if meaningful (i.e., information rich) temporal relationships are present on a timescale smaller than the scale of relevant temporal fluctuation of the stimulus. For example, it is difficult to imagine a mechanism used for encoding visual scenes that would be slower than the actual temporal accuracy of visual perception (VanRullen et al. 2005). If the same argument holds for any type of code, it must be used with care since, as already mentioned in the introduction, sensory systems and perception have multiple facets. Several circuits might implement in parallel several features of stimulus perception (e.g., novelty, noxiousness, quality, usefulness). Consequently, assessing the optimal perception and discrimination times of an animal to different stimuli might be used to define and put constraints on a minimal code that may be implemented by some part of a sensory system to compute simple behavioral responses. Addressing the question of the neural code used then leads to assessing which kind of perception or task this code might be used for.

Having this definition of temporal coding in mind, we will review, further in this chapter, different coding schemes in olfactory circuits that deal with temporal fluctuations of the neural activity. Some of them are temporal codes and others are not. To help the reader further in making up his/her mind about which coding schemes seem most plausible, we will first describe first what is known about the temporal constraints on olfactory perception.

#### **13.2.2** The Intrinsic Temporal Fluctuation of Smells

In a natural environment, odorant molecules are carried by air or water. These mediums undergo constant fluctuations, often incoherent and chaotic. Odors enter into contact with the olfactory receptor neurons (ORNs) depending on these fluctuations. Due to this constraint, the question is whether network activity is regulated by internal mechanisms, such as oscillations, or by these fluctuations. In this case, the olfactory system might have to encode this temporal feature in addition to odor identity and intensity information. It is noteworthy to emphasize an important difference. According to the temporal coding hypothesis, neurons might encode odor identity and intensity by generating a code that is temporally related. In the case of encoding fluctuations of the odor plume, the timing component is now carried by the stimulus itself and is therefore external to the system.

In consequence, as most experimental studies are performed using constant and long-lasting odor applications, particularly in nonbreathing animal models, we may wonder if an experimental bias has not been introduced. Nevertheless, some studies have specifically addressed the question of odor plume fluctuations. Vickers and colleagues (2001) have used an electroantennogram (EAG)-reflecting the activity of ORNs population-to monitor the activity of a moth antenna in response to pheromone plume fluctuations in a laboratory wind tunnel. The recorded preparation was moved around the center of the tunnel, changing its relative position in comparison to the pheromone plume wind flow. At low wind speed, the largest burst and the most variable EAG activity occurred in the central zone of the plume. As speed changed, the odor plume became more dispersed. The largest amplitude and the highest frequency fluctuations shifted to the periphery. The authors concluded that a very minor shift in the position relative to the odor plume can dramatically change the ORNs activity. They also performed intracellular recordings of PNs and observed that neurons are strongly time-locked to stimulus dynamics. The PNs firing was strongly correlated with EAG onset and their frequency of spiking increased with EAG bursts amplitude. Applying a varying odor plume in amplitude and duration, they observed a large range of the PNs frequency of discharge (0–150 Hz), indicating that activity is strongly dependent on the stimulus dynamics (Vickers et al. 2001). Other studies have suggested that the network dynamics are built to preferentially follow and encode these fluctuations. Christensen and colleagues presented a set of odor pulses at 1 Hz to the moth and recorded projection neuron (PN) single-unit activity (Christensen et al. 2000). They suggested that the PN firing pattern not only depended on the

chemistry, but also on the physical context. By presenting a blend of odor, they observed that two particular PNs responded in synchrony to a weak odor concentration, but displayed the complete contrary, a desynchronization when concentration was increased. These so-called emergent properties are thought to be strongly adapted to constantly changing odor plume (Christensen et al. 2000). A more recent study has also addressed the question of encoding fluctuating stimulus by the locust PNs (Brown et al. 2005). Intracellular recordings of the AL neurons and single-unit recordings of the AL and MB neurons have been performed. Varying numbers of odor pulses were applied at different frequencies. The authors analyzed the data at a neural ensemble level, monitoring the population activity by implementing a population vector analysis (see Figure 13.2A). Briefly, it consists of taking the number of spikes in a certain temporal window (= time bin), each vector row representing an individual neuron. The time-course of population activity is represented by a time series of the population vector. Authors have shown that independently of the number and frequency of pulses, the correlation between PN population vector activity of different protocols was high during some part of the odor presentation. These results suggest that the responses of multiple and rapid pulses are sufficiently correlated to one another to allow odor discrimination. Moreover, when pulses were brief, each new pulse-evoked activity truncated the previous one. Therefore, the network is adapted to be rapidly reset, allowing the next odor pulse to be encoded. Finally, authors have recorded the response of MB neurons (Kenyon cells, KCs) to such stimuli. They noticed that when the interpulse interval was brief, the largest response arose at the pulse onset, the frequency of discharge decreasing in the following pulses, and increasing again at the offset. This part of the olfactory system, at least in locusts, could therefore be more adapted to detect the odor appearance, the continuous odor flow fluctuations and its disappearance (Brown et al. 2005).

Brain oscillations have been proposed to be an important feature for odor discrimination (see Section 13.3). But oscillations sometimes develop with long-lasting odor application, which do not relate to the fast fluctuation timescales observed for odor plumes. Some studies have emphasized that these constant fluctuations may avoid the appearance of such oscillations (Christensen et al. 2000). In the moth, a study has described this phenomenon (Christensen et al. 1998). Intracellular recordings of the PN have been done, and 2 Hz pulsed as well as 5 s continuous odor stimulations were presented to the insect. The authors observed two different firing behaviors. When applying the pulses, no oscillations were observed and the authors suggested that information could be carried by a simple rate code. However, when the stimulus duration was increased, more temporally complex firing properties of PNs and oscillatory mechanisms emerged (Christensen et al. 1998). The same authors have also described that local field potential (LFP) oscillations and PNs spiking were not temporally correlated when applying brief odor pulses, questioning the role of oscillations in odor coding (Christensen et al. 2003).

In summary, these studies highlighted the importance of fluctuating odor plume in the olfactory coding. Neuronal population activity is able to track these dynamical changes with high precision. The coding strategy of the olfactory system may be strongly imposed by these dynamics. Theoretical work has suggested that temporally fluctuating stimuli are more reliable than constant ones, leading to more reproducible spiking behavior (de Ruyter van Steveninck et al. 1997). Experimental data tend to confirm this theoretical finding. Flying moths cease to make upwind progress and start to cast or counterturn across the wind as soon as they are exposed to a constant pheromone stimulus. This behavior may enhance the retrieval of the odor trace (Baker and Haynes 1989).

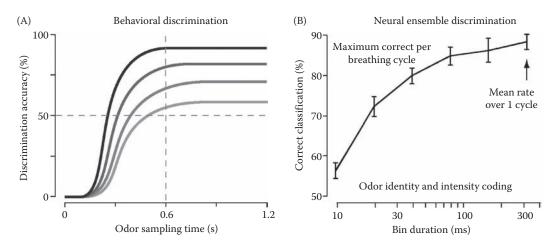
The results of higher efficiency of encoding fluctuating stimuli are subject to discrepancy. Other theoretical studies have shown that spiking variability of neurons in the fly visual system do not change between both conditions of stimulation, meaning that both are encoded with the same reliability (Warzecha and Egelhaaf 1999). However, these differences may be explained by variations among sensory systems. Different species that have a different ecological environment and different behavior may also need different coding strategies. Thus, a flying insect may need a system that can follow rapid fluctuation of the odor plume in order to quickly adapt and modify the flying trajectories, while a walking insect may need less rapid adaptation to follow an odorant trace.

#### 13.2.3 TIME AND OLFACTORY BEHAVIOR

Processing sensory inputs is performed with a certain delay due to receptor activation onset and transfer of information along sensory axons to the first brain relay. The subject of temporal coding brings up the question of the timescale needed for the olfactory system to segregate two odors. To address this question, researchers have used behavioral paradigms in mice and rats. Uchida and Mainen (2003) trained rats using an operant conditioning to perform two-alternative forced choices. They monitored the speed of discrimination between rewarded and unrewarded odors by video tracking. The rats had to discriminate between mixtures containing different proportions of two odors. First, the authors noted that the speed, but not accuracy, of discrimination is independent of mixture difficulty. In addition, accuracy increases with sampling time, but only up to 200 ms. A longer sampling time above 200 ms did not lead to further increases in accuracy, but, on the contrary, had the tendency to disimprove the performances. This study suggests that at 200 ms, the rat is able to discriminate between two odorants and discrimination time is independent of discrimination difficulty (Uchida and Mainen 2003). However, this two-choice discrimination task has some limitations. It measures the moment of head retraction, namely the motor command that makes the rat move from the odor port to the reward port. It has been claimed that the decision for such a movement and, more particularly, the olfactory discrimination process is taking place much earlier. Hence, some authors trained mice to a go/no-go task, which is thought to be more accurate for measuring the olfactory processing time. For an unrewarded odor, the mouse retracted its head and continued exploring the cage. However, for a rewarded odor, the mouse started licking the water reward. The head movement was tracked with a beam in the port. If odor was rewarded, the mouse stayed in the port and the beam was continuously interrupted. But, for the unrewarded odor, the beam was resealed when the mouse retracted its head. The speed of the discrimination task was determined by comparing both beam traces. The time point of head retraction (breaking point) significantly different from the time point of unbroken beam trace was defined as the decision time point. Authors have found a quite similar discriminating time for easy task meaning simple odors: ~200 ms. However, these authors have found a contrary result compared to Uchida. Indeed, they have noted that discrimination time significantly increased with binary mixture, even more with very close concentration of the two components (Abraham et al. 2004).

A more recent study seems to conciliate both views with the concept of speed-accuracy tradeoff (SAT), which has been described in both vision and audition. Authors claimed that both precedent studies might be biased by the fact that the mouse can choose the spending time in the port to better discriminate between two odors. Thus, they conceived a paradigm in which the odor sampling was chosen by the authors. They have observed that the accuracy depended not only on the difficulty of the task but also on the sampling time (Figure 13.1A). Indeed, the mice were more accurate when they were forced to discriminate with a longer odor sample. Hence, mice were able to discriminate in ~300 ms for the easy task, but the discrimination time reached ~600 ms for the hardest tasks (Rinberg et al. 2006). In terms of neural coding, these results would mean that more computational time is needed in the brain to differentiate odor-evoked neural responses and, therefore, make a decision. As such waiting more time allows collecting more information that could be used for increasing discrimination performances, as it can simply be observed by changing the size of the temporal window in which neuronal ensembles firing information is read (Figure 13.1B).

Experiments using freely moving animals might lead to some bias. First, behavioral paradigms generally measure the precise moment when the animal is moving as a discrimination time. However, such behavior implies other processes, like decision making and motor planning. These processes take time and olfactory discrimination might be processed upstream and earlier. Second, the onset of odor sampling is generally the instant when the valve opens to release the odor. Considering that the odor runs into the tubing, the exact odor-sampling onset can be biased by this delay. Finally, complex behaviors, such as decision making, are known to be influenced via feedback processes. Hence, some researchers have tried to sidestep this bias and addressed the question



**FIGURE 13.1** Speed-accuracy tradeoff in odor discrimination. (A) Relationship between odor discrimination accuracy and mouse sampling time during discrimination behavioral task. The curve is dependent of the task difficulty (increased difficulty indicated by progressive lightening of the gray colors). To reach the same accuracy, the sampling time must increase with increasing difficulty. (B) Odor classification prediction computed using information contained in the firing of the mitral cell population. Average of the maximum classification success measured in different breathing cycles when time bin duration is varied. The classification increases when population firing is considered for a longer time period. Error bars: SD across 15 breathing cycles after odor onset. ([A] Adapted from Rinberg, D., Koulakov, A., and Gelperin, A. *Neuron.*, 51, 351–58, 2006. [B] Adapted from Bathellier, B. et al. *Neuron.*, 57, 586–98, 2008a.)

of odor discrimination by reading the process time of rat OB using the calcium-imaging technique. The rat was head fixed and trained to an operant conditioning task, in which they associated an odor to a reward. The rat actively sniffed when they received the odor and started to lick if the odor was a rewarded one. Wesson and colleagues measured the delay between the onset of the first sniff and the odor-evoked calcium response (Wesson et al. 2008a, 2008b). Input arrived 100–150 ms after inhalation begin. Previous work by the same group has shown that the rat can discriminate an odor in a single sniff. The intersniff interval was ~75 ms, which is considered by the author as the central processing time of the OB to discriminate two odors. The total time of processing would then be 175–225 ms (Wesson et al. 2008a).

In conclusion, the consensus brought by these studies is that olfactory perception and discrimination occur very rapidly. The rodents are most likely using a single sniff to collect odor information in order to make a decision. Therefore, these behavioral data imposes temporal constraints that have to be taken into account in order to assess proposed odor codes.

#### 13.3 TEMPORAL DYNAMICS AT DIFFERENT TIMESCALES

#### 13.3.1 GAMMA AND BETA RANGE OSCILLATIONS IN THE OLFACTORY SYSTEM

#### 13.3.1.1 Phenomenology

Fast oscillations were probably the first example of temporal dynamics observed in the brain and, more specifically, in the olfactory system, described in the pioneering LFP studies of Adrian (1942, 1950). While recording in the OB of the anesthetized hedgehog, he noticed that prominent oscillatory variations of the extracellular potential occurred when the animal smelled an odor. These oscillations typically had a frequency in the range of 40–60 Hz, which is in the so-called gamma-frequency band. This observation has since been repeated several times in many mammals, both in anesthetized (Buonviso et al. 2003; Neville and Haberly 2003) and awake preparations (Kay and Laurent 1999).

It has also been shown that relatively fast oscillations are also triggered by odor stimulation in the OB of fish (Friedrich et al. 2004), in invertebrates such as the limax (Gelperin and Tank 1990), and in the insect AL (Laurent and Davidowitz 1994). Interestingly, the oscillation frequency is lower in fish and insects, reducing to around 20 Hz. This would correspond to the beta-frequency band if one follows the established nomenclature. However, 20 Hz oscillations in insects and fish are often referred to as gamma oscillations in analogy to the mammalian oscillations. Gamma-band oscillations were not only observed in the OB or AL, but also in downstream areas, such as the olfactory cortex in vertebrates (Freeman 1978) and the MB in insects (Laurent and Naraghi 1994). In rodents, careful analysis both in the OB and the cortex also revealed some odor-induced activity in the beta frequency band (15–40 Hz) (Boeijinga and Lopes da Silva 1989; Buonviso et al. 2003) in combination with gamma-band activity. Neither beta- nor gamma-band oscillations were ever described in ORNs.

Single or multiunit recordings performed in combination with LFP revealed that in insects (Perez-Orive et al. 2002), fish (Friedrich et al. 2004), and mammals (Buonviso et al. 2003; Eeckman and Freeman 1990; Litaudon et al. 2008), the action potentials of most principal neurons occur around a given phase of the extracellular oscillation. However, a synchronized neuron does not necessarily fire at gamma frequency and can skip several oscillation cycles (Bathellier et al. 2006; Friedrich et al. 2004; Lagier et al. 2004; Perez-Orive et al. 2002). In all cases, the preferred phase seems to be homogeneous among neurons, indicating that many neurons in the network fire in synchrony during the fast oscillations episodes (Eeckman and Freeman 1990; Friedrich et al. 2004; Laurent and Davidowitz 1994). Hence, the fact that gamma (or beta) oscillations are a collective behavior of a large number of neurons also explains why they can be observed in field potential recordings.

#### 13.3.1.2 Mechanism

Since the works of Rall and Shepherd (1968) and later Freeman (1975), it has been mainly hypothesized that fast oscillations are self-generated by the OB and the cortex due to strong inhibitory feedback loops (e.g., inhibitory interneurons pre- and postsynaptically connected to excitatory neurons). The self-generation hypothesis is supported by the absence of gamma oscillation in nasal epithelium activity and the fact that an isolated OB slice is able to generate gamma oscillation following even single and short olfactory nerve stimulation (Halabisky and Strowbridge 2003; Lagier et al. 2004, 2007).

Theoretical studies on neural networks endowed with inhibitory feedback loops have shown that, provided with a strong enough feedback and a large enough "delay" between firing and feedback, such networks can generate fast oscillations of their population firing rate. In this case, the network alternates between a period of decreased firing transiently imposed by inhibition and a period of increased firing where feedback inhibition builds up to start the next cycle (Bathellier et al. 2008b; Brunel and Wang 2003; Freeman 1975). Although other mechanisms, such as synchronization of oscillating neurons via gap junctions or excitatory synapses, can also reliably generate collective oscillations in neural networks, the inhibitory feedback loops provide a more robust mechanism. Importantly, it is not required that the neurons fire themselves at gamma frequency (Bathellier et al. 2006), which is, indeed, not the rule for the olfactory system neurons (Bathellier et al. 2008a; Friedrich et al. 2004; Perez-Orive et al. 2002). It has also been shown in artificial networks with inhibitory feedback loops that oscillation frequency remains stable when inhibition strength is globally changed in the network. This has been experimentally observed in slice preparations of the OB (Bathellier et al. 2006), but is not predicted by other oscillation generation mechanisms. Models of the insect AL also point toward a feedback loop mechanism that involves local interneurons (Bazhenov et al. 2001). In the OB of mammals, candidate interneurons for driving the gamma oscillation are the granule cells (Halabisky and Strowbridge 2003; Lagier et al. 2004, 2007), while it is hypothesized that the slower beta oscillations in the bulb are generated by a feedback loop originating from the olfactory cortex (Buonviso et al. 2003; Neville and Haberly 2003).

#### 13.3.1.3 Fast Oscillation and Behavior

The physiological relevance and importance of oscillations for sensory coding remain unclear. Oscillations themselves may be involved in odor coding mechanisms; on the other hand, they may just represent a side-product of the action of some inhibitory loops, which are themselves important for odor coding. It is evident that addressing this question is very important, but an experimental approach based on a pharmacological suppression of inhibitory loops to remove the oscillation would not succeed to simply clarify this point.

The fact that gamma-band (or beta-band) oscillations occur simultaneously with odor perception may suggest that they are in some way implicated in odor processing. This idea was further supported by the observation that the amplitude of gamma-band activity over the OB is heterogeneous and that its spatial distribution depends on presented odors and behavioral contexts (Freeman and Schneider 1982). It was recently established that the power of gamma-band oscillations in the bulb increases in behaving animals when they have to perform difficult olfactory discriminations (Beshel et al. 2007). In the insect, suppression of fast inhibitory feedback by picrotoxin has been shown to suppress gamma oscillations and to deteriorate olfactory performance (Stopfer et al. 1997). It has also been observed in the rat that the relative power of gamma-band and beta-band oscillations can change dramatically with the animal's experience. While novel perception of an odor is mainly associated with gamma-band activity in the bulb and cortex, beta-band oscillations become more prominent when the animal has learnt this odor (Ravel et al. 2003).

However, these results cannot yet help in deciding whether an increase or decrease in oscillation amplitude is the consequence of the differential involvement of some inhibitory feedback mechanisms, or correspond to a direct role of the oscillation in olfactory coding. Further work is needed to answer this important question.

#### 13.3.2 Oscillatory Dynamics due to Sniffing in Mammals

#### 13.3.2.1 Phenomenology and Mechanism

Other prominent neuronal oscillations observed in the olfactory system of terrestrial vertebrates are the slow oscillations synchronized to the animal's respiratory cycle. In rats and mice, these oscillations range between 2 and 10 Hz, depending on the animal's sniffing behavior, and can be clearly separated from gamma and beta oscillations. Unlike gamma and beta oscillations, these slow oscillations are present in all three main stages of the olfactory system: in the sensory neurons (Spors and Grinvald 2002; Spors et al. 2006; Verhagen et al. 2007; Wachowiak and Cohen 2001), in the OB (Bathellier et al. 2008a; Buonviso et al. 2003; Cang and Isaacson 2003; Macrides and Chorover 1972; Margrie and Schaefer 2003; Onoda and Mori 1980), and in the olfactory cortex (Rennaker et al. 2007). They can be observed, although with a weaker amplitude, even in the absence of any odor input, but are absent when animals are tracheotomized and do not breath via the nose (Onoda and Mori 1980). Conversely, imposing an artificial sniffing cycle via a tube plugged in the trachea and directed to the nose is sufficient to induce slow oscillations in the olfactory system at desired frequency. Hence, the slow oscillations originate from the constant modulation of airflow at the nasal epithelium, leading to sensory neurons mechanical activation (Grosmaitre et al. 2007), but are not due to a synaptic drive internal to the brain.

The slow oscillations are clearly visible in both the LFP recordings (Buonviso et al. 2003) and in the firing patterns of receptor neurons (Duchamp-Viret et al. 2005), mitral cells (Bathellier et al. 2008a; Buonviso et al. 2003; Macrides and Chorover 1972; Onoda and Mori 1980), and pyramidal cells of the olfactory cortex (Rennaker et al. 2007). It is a global modulation of neural activity. However, different olfactory receptors can fire at different phases of the respiratory cycle (Spors et al. 2006). Likewise, different mitral cells can fire at different phases of the breathing cycle in the OB (Bathellier et al. 2008a), but the same mitral cell can also exhibit different phasing, depending on the odor and concentration presented to the animal (Bathellier et al. 2008a; Macrides and Chorover 1972). In general, several spikes are fired in a breathing cycle, but the number of spikes and their timing are extremely variable across cells and presented odors, giving rise to a large diversity of temporal firing patterns (Bathellier et al. 2008a).

At a population level, as has been recently shown for mitral cells, the result of this diversity is the emergence over time of complex neuronal ensemble activation patterns (Bathellier et al. 2008a). Different snapshots of the mitral cell population activity at different time points of the breathing cycle can be as dissimilar from each other as two snapshots taken during presentation of different odors (see also Section 13.3.3). Similar conclusions can actually be drawn for ensembles of ORNs derived from calcium imaging of glomeruli responses (Spors et al. 2006). Hence, unlike gamma oscillations, temporal complexity at breathing frequency originates, at least in part, from odor detection and transduction mechanisms themselves. Airflow, odor adsorption, and receptor dynamics might therefore play a significant role (Scott et al. 2006). In the bulb and cortex, internal network dynamics might also shape temporal patterns of principal neuron activity. However, because no thorough comparison of cyclic dynamics at different levels of the olfactory system has been conducted yet, it is hard to evaluate the respective contribution of intrinsic and extrinsic mechanisms in these dynamics.

#### 13.3.2.2 Breathing Cycle Dynamics and Behavior

If many studies of the breathing cycle dynamics have been carried out in anesthetized animals, for which sniffing frequency is rather constant, one must not forget that behaving animals constantly adjust their sniffing (Kepecs et al. 2007). Odorant inflow in the nasal cavity via sniffing is therefore an extraordinary generator of temporal variability (although under the control of the animal) rather than a precise clocking system. Some results, developed in Chapter 12, show that sniffing frequency can strongly change the patterns of inputs to the OB (Verhagen et al. 2007). This must be taken into account when defining any theory evaluating the role of breathing cycle dynamics in olfactory coding. In addition, a wide range of sniffing frequencies should be explored at each level of the olfactory system in order to be conclusive.

Even if interaction between respiration and sense of smell is only relevant for part of the animal reign, the olfactory system of the other species has also to deal with temporal fluctuations of the odor input. In some cases, fluctuations can also be actively controlled, as for some arthropods able to oscillate their antennae during odor perception (Koehl et al. 2001). More generally, the fluctuations are imposed by the structure of odorant stimuli, which contact odor sensors in a temporally discontinuous fashion. Interestingly, the response of AL neural ensembles to oscillating inputs are very similar to the rapidly evolving cycle observed in the rodent OB (Brown et al. 2005). This suggests a broad relevance for the study of slow oscillating or fluctuating dynamics (2–10 Hz frequency range) in most olfactory systems.

#### 13.3.3 SLOW, NONOSCILLATORY PATTERNING IN FISH, INSECTS, AND MAMMALS

#### 13.3.3.1 Phenomenology and Mechanism

There is a third type of temporal dynamics in the olfactory system. When a step odor stimulus is given to an animal, recorded neural responses are usually not following a stepwise time-course, even in the absence of breathing or if one averages neuronal activity over each breathing cycle. Instead, many neurons exhibit strong changes in their firing rate over time after odor onset and offset. For example, some neurons can be first inhibited and start firing with some delay, but the opposite is also possible. These changes are robust across trials. This was first evidenced in the OB of tracheotomized rats (Meredith 1986). Later on, complex temporal fluctuations of individual PN firing activity were shown in the insects AL (Laurent 1996; Wilson et al. 2004) and in the fish OB (Friedrich and Laurent 2001). In order to overcome the diversity of single neuron responses and try to better understand odor coding, some studies have analyzed odor responses

in neural cell assemblies. To do so, the firing responses of a large number of recorded neurons can be simultaneously considered by putting them together in a population vector (Figure 13.2A). As mentioned earlier, it consists of taking the number of spikes in a certain temporal window (= time bin), each vector row representing an individual neuron. Every vector, therefore, represents a snapshot of the neuronal population activity in a defined time bin. It is important to note the difference with an average population firing, as population vectors preserve the specificity of individual neural responses. The time-course of population activity is represented by the time series of the population vector, which can be plotted as trajectories in a multidimensional space (space of all recorded neurons) (Figure 13.2B).

This analysis was first conducted in the fish OB, demonstrating that the activity of mitral cell ensembles could significantly change over a period of at least 1 s after steady odor application onset (Friedrich and Laurent 2001). In the locust AL, population activity is in a resting state that is left

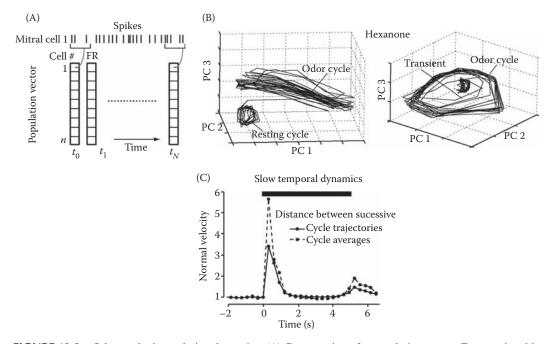


FIGURE 13.2 Odor-evoked population dynamics. (A) Construction of a population vector. For any time bin  $(t_0 \text{ to } t_N)$ , the *n*th dimension of the vectors corresponded to the average firing rate (FR) of the *n*th-recorded cell. (B) Average trajectory of the population vector (eight bins per cycle, from -2.5 to 4 s relative to odor onset) before and after hexanone 0.20 application and visualized in PCA space. Two different viewing angles are shown. Two types of dynamics are visible: slow dynamics reflecting changes of firing across respiratory cycles, and fast pseudocyclic dynamics reflecting breathing cycle internal dynamics. (C) Comparison of highand low-resolution descriptions of slow temporal dynamics. Population vector trajectories become stable after going through a slow transient. The rapidity of this process was measured by computing the velocity of the population vector averaged over 312 ms time bins (i.e., mean breathing cycle duration). However, it is conceivable that the cyclic trajectories themselves converge with a different timing as their average. In this figure, the distance between successive cycle trajectories (a cycle trajectory represented by the concatenation of its decomposition in eight population vectors) and successive cycle averages (i.e., the average population vector over a breathing cycle) are plotted. All points are normalized by the observed baseline value. These velocity measures indicate that the convergence of cycle trajectories occurs within the same time period as their average (roughly 1 s to reach a steady state). Fitting a single exponential function on the curves also yields similar time constants (386 ms for the cycle average and 499 ms for the cycle trajectories). Therefore, both convergences probably result from the same dynamical process. ([A-C] Adapted from Bathellier, B. et al. Neuron., 57, 586-98, 2008a.)

after odor onset, and typically evolves during roughly 1 s and then settles in a steady state called a fixed point, which lasts until the end of the stimulus application. Thereafter, it takes approximately 1 s for the population activity to settle back in its resting state (Mazor and Laurent 2005). A similar phenomenon was also recently observed in the mouse OB, superposed with the breathing cycle oscillation (Bathellier et al. 2008a) (Figure 13.2B). During the first second of odor presentation, population activity changed from cycle to cycle (Figure 13.2C), and after this initial period, population activity repetitively described the same cycle as long as the stimulus was sustained. These dynamic transitions could be better visualized when computing the rate at which the vectors changed over time (i.e., vector velocity, representing the distance between the population activity kept evolving significantly above "noise" (i.e., baseline velocity) for ~1 s, and then reached a steady state. Another transient evolution was observed at odor offset and then the population settled in a poststimulus state that slowly drifted back to the resting state (within ~ 20 s).

A slow convergence of the firing rate has also been observed in fish (Friedrich and Laurent 2001) and in insect (Hallem and Carlson 2006) receptor neurons. However, at this level, all neurons display the same phasic time-course contrary to the OB or AL principal neurons, which exhibit various time-courses. The complexity of the response can be quantified by computing the correlation of the series of population vectors with the first vector after odor onset. The correlation stays very close to 1 for a population of receptor neurons, and can dramatically drop close to 0 for OB or AL neurons. It must be noted here that the decrease of the correlation is less pronounced in the mouse OB (Bathellier et al. 2008a), than in the fish bulb (Friedrich and Laurent 2001) and locust AL (Brown et al. 2005; Stopfer et al. 2003). This indicates a reduced complexity of slow, nonoscillatory dynamics at population levels in mammals.

The mechanisms of the slow nonoscillatory dynamics have not been clearly established yet. In ORNs, the phasic response profile seems to correspond to a rate adaptation mechanism. These input dynamics should play a role in the dynamics observed at the next level, although the increased complexity there suggests the involvement of other mechanisms, such as synaptic interactions. It was shown in the AL of insects that the GABA<sub>A</sub> receptor antagonist has no effect on slow temporal dynamics (Stopfer et al. 1997; Wilson and Laurent 2005). On the contrary, GABA<sub>B</sub> antagonists seem very potent in reducing the complexity of single neuron temporal firing patterns (Wilson and Laurent 2005). Hence, slow inhibitory synapses in the AL probably contribute substantially to the slow dynamics, as also suggested by theoretical work (Bazhenov et al. 2001). It is not known to what extent these results can be applied to the OB.

#### 13.3.3.2 Slow Dynamics and Behavior

The impact of slow convergence of neural activity on olfactory perception has never been directly studied. Nevertheless, comparison of perceptual delays with the 1 s time constant typically observed for the slow dynamics of neuronal activity can help set some constraints on the potential role of the latter phenomenon. Mice and rats were observed to discriminate between two odor pairs within 100–500 ms (Abraham et al. 2004; Uchida and Mainen 2003). This indicates that full convergence of neural activity to its steady state may not be required for perception. However, a study in mice suggests that discrimination of close odor pairs requires longer delays that for simple odor pairs. Therefore, one could imagine that slow convergence has a role in improving the discriminability of two odor percepts. This question will be discussed further in Section 13.4.3.

#### 13.3.4 SHORT-TERM PLASTICITY IN INSECTS

Up to now, we have reviewed temporal schemes that shape neuronal activity in the olfactory system at different, clearly separable timescales: fast (gamma and beta from 20 to 80 Hz), intermediate (breathing cycle, 2-10 Hz), and slow (slow convergence,  $\sim 1$  Hz). A fourth phenomenon, only observed in the locust, should be mentioned to end this list. When several pulses of an odorant stimulus are

successively presented to a locust, neuronal responses change drastically from one pulse to another (Stopfer and Laurent 1999). The amplitude of gamma oscillation in the AL increases, while the number of PN spikes decreases, but their temporal coherence and accuracy increases. These modifications are not due to receptor neurons, and should be intrinsic to the AL neural circuits. The characteristics of these modifications (decrease of activity, increase in synchrony) suggest that they could originate from an increase in the strength of some inhibitory feedback loops, which is known to produce exactly the same changes in networks similar to the AL (Bathellier et al. 2006). Interestingly, discontinuous odor inputs are more potent than continuous inputs for triggering changes in the neural response. The phenomenon is quite insensitive to the interval between odor pulses (in the range of 2.5–20 s) or to the duration of these pulses (0.2–2 s). Unfortunately, it is not known how the system behaves when odor pulses become temporally very close, as occurs, for example, in an actively sniffing mammal. The phenomenon is also odor-specific, suggesting that it is a form of odor memory. But the "memory" of the received odor lasts no longer than 10 min. However, to the best of our knowledge, this peculiar form of temporal dynamics has never been described in other species.

# 13.4 CODING HYPOTHESES LINKED TO TEMPORAL DYNAMICS

#### 13.4.1 TEMPORAL CODING WITH THETA OR GAMMA FREQUENCY

Observation of temporal dynamics in neural responses is a fact, but their significance and contribution to odor coding is still unclear. Some hypotheses have been described in coding schemes that only use temporal features to build the odor code and discard classical measure of neural activity, such as firing rate or spike counts. Even if, in light of recent experimental evidences, these hypotheses appear as such rather unlikely, they contain key concepts that could be involved in more complex theories and are worth presenting in this chapter.

#### 13.4.1.1 Coding with Spike Timing in the Breathing Cycle Oscillation

As demonstrated by Hopfield (1995), oscillations are ideal to build up temporal codes because a neuron driven by an oscillatory current fires with a timing that varies with the amplitude of its input. This makes it possible to transfer any analog information (e.g., odor concentration) via another analog variable (spike timing), thereby avoiding the loss of precision induced by spike count coding schemes (discrete variable).

Studying mitral cell responses to odors from in vivo patch-clamp recordings, Margrie and Schaeffer (2003) found that the latency of the first spike fired in a breathing cycle is dependent on odor identity and intensity. The authors also found that the number of spikes per cycle depended on odor identity and intensity, but not interspikes intervals, which led them to conclude that the instantaneous firing rate of a cell cannot efficiently code for the stimulus. They proposed instead that spike latency could encode the stimulus much better. Their claim was further supported by a simple computational model in which latency-based coding was shown to have a larger capacity (i.e., number of odors that can be encoded with a given number of neurons) than spike count or instantaneous firing-rate-based coding. However, more recently, a direct measure of the efficiency of latency-based coding in the mouse OB showed that contrary to the prediction of Margrie and Schaeffer (2003), it is much less efficient than a firing-rate-based coding (Bathellier et al. 2008a). Two explanations for this result can be given. First, latency does not systematically vary with odors and concentrations. There is a significant negative correlation between concentration and firing latency, but it appeared to be rather loose (Bathellier et al. 2008a). Second, the precision of firing latencies from trial-to-trial is rather poor, which directly limits the precision of the coding scheme. Noise constraints were not taken into account in the model proposed by Margrie and Schaeffer (2003), explaining why they could find better performance for spike latency-based coding. In physiological noise levels, the first spike latency is very variable. Therefore, a coding based on this sole parameter would not be very efficient and seems rather unlikely in the OB.

#### 13.4.1.2 Coding with Synchrony in a Neural Population

Another key phenomenon that can be used in a temporal coding scheme is synchrony between different neurons. As mentioned earlier in this chapter, gamma oscillations observed in the LFP are due to synchronous activity of many neurons. It has long been recognized that synchrony can be a way of transmitting information across neural networks, and many experimental studies support the idea that the brain might make great use of synchrony (Varela et al. 2001).

Recently, Brody and Hopfield (2003) have proposed an odor-coding scheme for the OB based solely on synchrony (Figure 13.3A). This scheme uses the fact that in a population of integrate-and-fire neurons receiving the same oscillatory drive and firing roughly at the same frequency, there exists a range of input current amplitudes in which neurons are well synchronized. When an odor enters the nose, a specific group of mitral cells would receive currents that are in the appropriate range and would be synchronized. Other cells would still fire asynchronously. Synchronized neurons could then be easily detected by downstream neurons or neuronal circuits endowed with appropriate bandpass filtering properties (e.g., circuits with delayed feed-forward inhibition are bandpass filters).

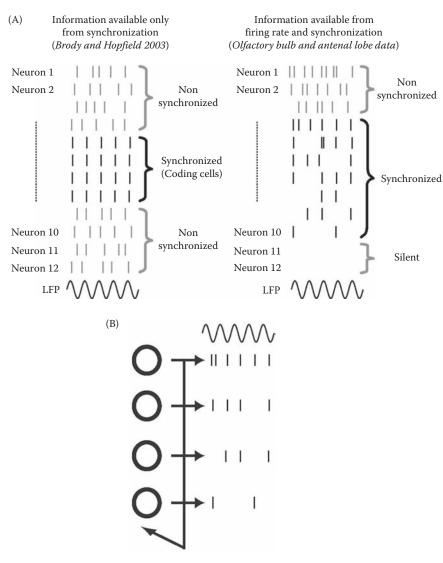
The interesting aspect of this theory is that mitral cell assemblies could encode odor information without changing their firing rate. However, it has now been clearly demonstrated that in insects (Stopfer et al. 2003), fish (Friedrich and Laurent 2001), and mammals (Bathellier et al. 2008a), mitral cells actually do change their firing rate during odor stimulation, and that firing rate changes carry odor information. Therefore, the reality of the olfactory system is more complex than the simple model of Brody and Hopfield, and it is, in fact, unlikely that synchrony alone carries odor information in the olfactory system. In order to match experimental observations more closely, several theories have tried to conciliate firing rate changes and neural synchrony in a single coding scheme. We review some of them in the following sections.

### 13.4.2 GAMMA SYNCHRONY: CLOCKING OF NEURONAL INTEGRATION

The role of neural synchrony in the gamma frequency band has been particularly emphasized in the insect olfactory system, mostly in the locust and in the honeybee. One hypothesis that is now supported by several pieces of evidence is that each cycle of the gamma oscillation serves as a time window for integration of upstream activity by downstream networks.

As described in Section 13.3, gamma oscillations (~20 Hz) appear in the AL, where they are most probably intrinsically generated due to inhibitory feedback loops (Figure 13.3C). Odordependent neural activity in the AL is also very dynamic on a slower timescale with a transient lasting up to 1 s, during which ensemble activity is strongly reshaped in time. Due to these temporal dynamics, it is crucial to know on which timescale activity is integrated by downstream networks. Perez-Orive and colleagues (2002) have suggested that integration of AL activity by KCs in the MB occurs over single cycles of the gamma oscillation. The proposed mechanism is the following. Both AL PNs and KCs tend to synchronize on the gamma cycle. On average, PNs lead KCs by almost half a cycle, a delay that probably corresponds to the propagation of spikes from the AL to the MB. A population of inhibitory neurons in a separate region of the insect brain (lateral horn) also receives feed-forward excitation from PNs and fires roughly at the same phase of the gamma oscillation as the KCs. Lateral horn inhibitory neurons project onto the KCs, sending a strong barrage of inhibition that arrives in the last part of the cycle. This inhibitory input prevents further firing of the KCs and tends to reset integration of excitatory inputs before the next cycle begins, suggesting that the integration time window of the Kenyon cell (KC) only spans a gamma cycle. The consequence is that all temporal features of AL activity slower than 20 Hz can theoretically be retained by the MB.

The role of feed-forward inhibition in the MB is not restricted to the establishment of a precise integration window. It also drastically reduces the firing of KCs (Perez-Orive et al. 2002), contributing to the transformation of a dense representation odor in the AL (i.e., each PN responds



Inhibitory feedback = Oscillations + firing rate regulation

**FIGURE 13.3** Oscillations and neuronal synchrony in odor coding. (A) Comparison between the temporal coding scheme proposed by Brody and Hopfield (2003) and typically observed firing in the olfactory bulb or the antennal lobe. In the Brody and Hopfield model, all neurons have roughly the same firing rate, but a sub-population fires synchronously, coding for a specific odor. In typical olfactory bulb or antennal lobe data, both the firing rate and the degree of synchrony vary between neurons, so that both may encode odor information. (B) Synchrony may arise in a neural population due to an inhibitory feedback loop. This loop also regulates the firing rates of individual cells.

to many odors) into a sparse representation in the MB (i.e., each KC responds to few odors; Perez-Orive et al. 2002). Indeed, a pharmacological block of inhibition in the MB considerably reduces sparseness. Sparse representations are thought to offer many advantages for memory storage in neural networks. Hence, the feed-forward inhibition from the lateral horn is probably a crucial mechanism for efficient olfactory function. It is, however, not clear whether the fact that it occurs in a rhythmic fashion is crucial to MB function or whether it is just the consequence of

the presence of inhibitory loops in the locust olfactory system that downregulates activity and generates an oscillatory behavior.

Recently, some experimental evidence started to provide an answer for this question. It was recently demonstrated that gamma synchrony is actively transferred across the synapse between KCs and their downstream targets (the beta-lobe neurons) by a spike timing-dependent plasticity mechanism (Cassenaer 2007). This mechanism acts so that beta-lobe cells, which fire too late with respect to the gamma cycle, have their synaptic input increased so that they fire earlier, and conversely. The existence of such a mechanism supports the idea that the clocking of neural activity by gamma oscillation is important for the function of the olfactory system. It is, however, not a definitive proof. Spike timing-dependent plasticity is a general mechanism that is thought to underlie some of the learning phenomena in the nervous system (Kepecs et al. 2002). If some theoretical studies have shown that it mechanically increases synchrony of neural activity (Suri and Sejnowski 2002; Zhigulin et al. 2003), many other studies point out other crucial functions, such as optimization of information transfer (Toyoizumi et al. 2005). Currently, it is not possible to rule out that spike timing-dependent plasticity is present in this synapse to fulfill one of these other functions and that, as a byproduct, it also sharpens gamma synchrony.

#### 13.4.3 SLOW DYNAMICS AND DECORRELATION

As we have detailed in previous sections, aside from gamma oscillations, olfactory system activity also exhibits slow temporal patterns. The idea that integration across different stages of the system might occur at the timescale of a gamma cycle would imply that slower patterns can be entirely read out by downstream structures. Nonetheless, the function of slow temporal patterning is not yet clear. We have mentioned earlier that coding solely based on spike timing with respect to the breathing cycle oscillation was unlikely. The breathing cycle is also not present in all species. But what about the slow, nonoscillatory dynamics of neural activity, which is conserved across phyla?

Slow dynamics are, in some form, present in all stages of the olfactory system, but gain in complexity in the OB or equivalently in the AL. In both networks, these dynamics are characterized by a reshaping of ensemble activity over time, which ends roughly 1 s after odor onset (Bathellier et al. 2008a; Mazor and Laurent 2005). One possibility that we will explore in the last section is that this slow patterning builds a temporal code, and that the entire trajectory of neural population activity is needed to determine which odor the animal received. But another interpretation would be that slow dynamics reshape neural activity to perform some kind of processing of the odor information.

An hypothesis along this line was proposed by Friedrich and Laurent (2001). It was observed that in the fish OB, ensemble activity evolves in time toward less correlated and thereby more easily separable representations of different odors. This result was obtained by computing the correlation between all population vectors representing the ensemble response to a set of amino acid odors. It shows that the mean correlation decreases progressively in time after odor onset, taking approximately 1 s to reach a minimum. The odor classification success based on population vectors also increased in time. Interestingly, this outcome was not observed in a population of receptor neurons (Friedrich and Laurent 2001), suggesting that the improvement of odor representations is generated by the bulb circuitry. Hence, it was proposed that slow dynamics in the OB aims at improving the discriminability of odor percepts.

Surprisingly, somewhat dissimilar results were obtained in the locust AL. Indeed, in the locust, the PNs response to long odor pulse decreases in precision over the same time period ( $\sim$ 1 s), while the population converges to a fixed point of the dynamics (Mazor and Laurent 2005). The similarity between population vectors representing responses to different odor also increases. This suggests that slow dynamics in the locust produces opposite effects to those observed in fish. Careful analysis of locust ensemble activity, however, shows that representations decorrelate from 0 to  $\sim$ 100 ms after odor onset, as observed in fish on a much longer timescale. The discrepancy in decorrelation speed makes it questionable whether the two phenomena are comparable. It is,

however, possible that slow dynamics, which develop both in fish and insects over the timescale of 1 s and yield comparable temporal patterns of neural activity, would represent different speciesspecific mechanisms. In mice, current data show that discriminability between representations of different odors in the OB does not improve on the slow timescale, but does not decrease either (Bathellier et al. 2008a). To this end, care should be taken with the interpretation of these results. Discrepancies exist in the experimental conditions and in the set of stimuli tested, which could also explain the apparent contradictions. It should be mentioned, for example, that mice experiments were performed in freely breathing animals, for which breathing cycle dynamics interact with the slow convergence of ensemble activity. In insects receiving fluctuating inputs so that ensemble activity of PNs does not reach a fixed point, no decrease of discriminability has been observed (Brown et al. 2005). Careful parallel analyses remain to be done to decide whether the impact of slow dynamics is really dissimilar in different species. Overall, slow dynamics appears as a complex phenomenon whose purpose and mechanism are far from being resolved yet.

# 13.4.4 MULTIPLEXING: COMBINING SLOW AND FAST DYNAMICS

Even more complexity can be added if one combines fast and slow dynamics in the odor code. It was recently shown that the temporal behavior of odor representations in the fish OB depends on phase locking of spikes to the ensemble oscillation (Friedrich et al. 2004). In fish, spikes that are not phased-locked to gamma oscillations turned out to be the majority. Considered separately, they form neural representations that decorrelate over time, as described in the previous section for an unsorted population. On the contrary, the minority of phased-locked spikes yields ensemble representations that become more correlated over time.

This observation led the authors to propose that phased-locked spikes carry information about odor categories that is lost in non-phased-locked spikes during the decorrelation process. In other word, two codes could be used in parallel. A firing-rate-based code for odor identity, and a synchrony-based code for odor categorization. This interesting hypothesis on how different codes might serve different purposes in the same neural system deserves to be explored further, in particular to test whether downstream targets of mitral cells in fish actually make use of the two codes.

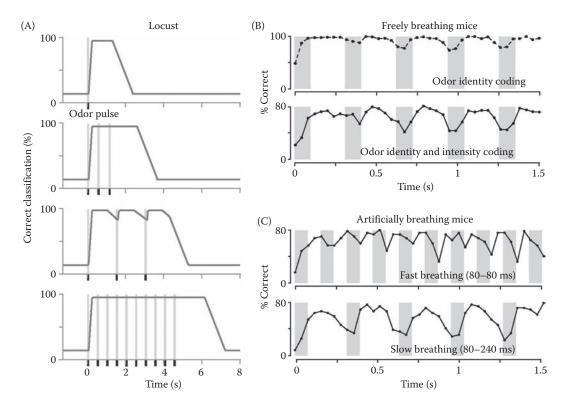
# 13.4.5 INFORMATION CONTAINED IN TEMPORAL SEQUENCES

If the response of a population of neurons to odors has the form of a complex temporal sequence, there are three alternatives for coding:

- Neurons firing rates in a single and fixed time window of the sequence could be used for read out, while other time bins of the sequence would just represent elements of the brain dynamics that lead to the read out time bin. This is the simplest scheme, which is used in most artificial sensor systems.
- Different time windows could equivalently be used for reading out firing rates. The brain could "choose" the time window to adapt to the requirement of the tasks it is engaged in (e.g., earlier time window for speed, best time window for accuracy).
- The combination of all spike times in the sequence could be read out during a certain time window (whose duration could be flexible). This temporal scheme is equivalent to counting the number of spikes falling in successive time bins, with the duration of the time bin representing the accuracy of the spike time measure.

As long as the "decoding" mechanisms in brain networks downstream to the considered population are not known, it is hard to figure out which scheme this population actually implements. But, some hints can be obtained from the analysis of the structure of the neural signal. The first possibility would be evident if information about odors was clearly concentrated in a specific time window. The second and third possibilities would, on the contrary, require the neural response to be informative on several time windows. The third possibility would be more likely if spikes time-based coding was more informative than firing-rate-based coding on the same time interval.

Recordings from neuronal populations in the insect AL (Brown et al. 2005; Mazor and Laurent 2005; Stopfer et al. 2003) and the fish OB (Friedrich and Laurent 2001) indicate that enough information about the stimulus is present in the firing rates of 50–200 cells to discriminate between eight and 16 odors with less than 20% error in any time bin of 50–400 ms from stimulus onset to 1 s after stimulus offset (Figure 13.4A). Here, the effect of time bin duration was not studied. It is clear that all time bins contain non-negligible information about the stimulus. Of course, as mentioned earlier, some time bins contain a bit more information than others. They are found 1 s after odor onset in fish, and in 100–200 ms to 1 s after odor onset or offset in locusts (Mazor and Laurent 2005). Data, therefore, indicate that different time bins can be used for decoding. The same qualitative outcome was obtained in the mouse OB (Bathellier et al. 2008a) (Figure 13.4B and C): all breathing cycles (a cycle represents ~300 ms in this study) following odor onset were equally informative (~10% error for 101 cells). However, within a breathing cycle, the prediction error for time bins of ~40 ms



**FIGURE 13.4** Neuronal ensembles coding and input dynamics in locusts and mice. (A) Ensemble responses can be used to classify the set of odor trials presented to the animal, regardless of the change in the odor application patterns (indicated by the gray bars and black boxes on abscissa). For this dataset, chance level was 12.5%. (B) Classification performance over time for odor identity only (*top*) or for odor identity and intensity (*bottom*) in freely breathing animals. Note that similar maximum classification is reached across different cycles. Gray rectangles indicate inspirations. (C) Classification performance over time for odor identity and intensity in artificially breathing animals. Increasing the frequency of breathing is not changing the maximal classification performance across cycles but when it occurs. ([A] Adapted from Brown, S.L., Joseph, J., and Stopfer, M., *Nat. Neurosci.*, 8, 1568–76, 2005. [B, C] Adapted from Bathellier, B. et al. *Neuron.*, 57, 586–98, 2008a.)

could vary from 20 to 60% for 15 odorant stimuli (chance level = 93.7% prediction error). This is a quantitative difference to locusts or fish, which do not show such large and rapid variations of prediction error. It is interesting that in mice, time bins that are preferential for decoding appear only at a fast timescale. In the mouse bulb, progressively increasing the time bin duration from 40 ms to one breathing cycle (300 ms) could monotonically decrease the prediction error from ~20% down to ~10% (Figure 13.1B, Bathellier et al. 2008a). This shows that averaging out rapid temporal fluctuations actually produces no loss of information for a population code. Instead, it increases read out information because time averaging reduces noise levels.

To date, the information contained in true temporal coding schemes has only been evaluated in mice (Bathellier et al. 2008a). In mice, the strongest changes in population activity are observed within breathing cycles. When breathing cycles were divided into 16 time windows to describe the temporal sequence of spikes, the combined information of all 16 time windows yielded a prediction error of 8% for 15 odor stimuli and a population of 101 mitral cells. In comparison, the mean firing rate computed over one breathing cycle yielded 13% prediction error. Hence, for the 15 odorant stimuli of this study, the information contained in the full temporal sequence of population activity added little to the information that could already be extracted from mean firing rates. In addition, most of the temporal information was contained in slow temporal features. Odor prediction based on the first Fourier coefficient of the activity sequence (i.e., mean phase and modulation amplitude of neuronal activity in a breathing cycle) yielded only 9% error, but the error dramatically increased when subsequent Fourier coefficients (i.e., finer temporal features) were used alone for odor prediction. Hence, some temporal information is clearly present in OB activity in mice. Current data are, however, not sufficient to decide whether this information is really useful to the system, because for the small number of odorant stimuli tested, roughly the same performance was obtained whether temporal information was used for read out (sequence) or not (average firing rate). It is clear from existing data that temporal information is not necessary for simple odor discrimination. But temporal information could improve olfactory coding in two different ways. Either it could bring more sensitivity, helping in difficult odor discriminations, or it could increase the information capacity of the olfactory system, allowing encoding of more odors with less neurons. Further investigation should be carried out to address these questions.

# 13.5 CONCLUDING REMARKS

Recordings of neurons in the olfactory system have now highlighted the diversity and temporal complexity of odor-evoked firing patterns. In this chapter, we endeavored to give an overview of the temporal dynamics that has been observed so far. It is interesting to note that the published studies support analogies in the dynamics of odor representations in evolutionary very different species, such as mammals and insects. It is, therefore, tempting to think that these dynamics may be underlying for some part of the odor code, at least in the first relay of the olfactory system. However, this remains a hypothesis that has to be carefully tested. Indeed, as long as the "decoding" mechanisms in downstream brain networks are not better known, it will be hard to figure out which encoding schemes are used. The challenge for the next years will be to record neural activities in different networks of the olfactory system and try to link them to the animal behavior. In addition, unraveling the molecular, cellular, and network mechanisms underlying the different observed dynamics should help us understand what are the actual coding principles used in the brain in olfaction and assess whether they are optimal.

# ACKNOWLEDGMENT

We thank the University of Geneva and the Swiss National Science Foundation for their financial support.

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# 14 Cortical Activity Evoked by Odors

Donald A. Wilson and Robert L. Rennaker

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# 14.1 INTRODUCTION

It has been hypothesized (Lynch 1986; Aboitiz et al. 2002; Montagnini and Treves 2003) that the mammalian cortex initially evolved as an associative structure, allowing features of the sensory world extracted by more peripheral circuits to be merged both within and between sensory modalities into objects capable of driving behavior. Associative cortical circuits generally have broadly distributed, overlapping inputs, allowing convergence of different pieces of information. This is in contrast to classic topographic, hierarchical cortical circuits, where information flow is more restricted to narrow, specialized channels, with much less cross-talk between disparate inputs.

The early mammalian cortex, like the modern reptilian cortex, was dominated by olfaction (lateral cortex) and hippocampus (medial cortex), with a multimodal interface (dorsal cortex) between the two. The olfactory cortex and hippocampus are characterized by nontopographic, associative networks capable of merging distributed, diverse, collections of inputs into everything from rich memories of specific life events, to maps of the visuospatial world, to single olfactory percepts derived from complex molecular mixtures. Only with continued evolutionary expansion of the cortex through the emergence of the neocortex did regional specialization and topographic, unimodal sensory processing come to be expressed, as seen, for example, in the mammalian primary visual or auditory cortex (Lynch 1986; Montagnini and Treves 2003).

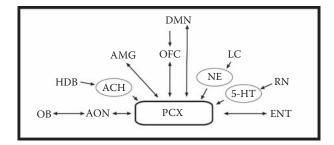
Thus, the strongly associative nature of the primitive cortex—i.e., trilaminar cortices like the piriform cortex or hippocampus—promotes synthetic object processing, as opposed to analytical processing of features from complex mixtures. The processing of complex stimulus patterns as objects by associative circuits leads to robust stimulus recognition in the face of degraded inputs and enhanced discrimination of overlapping patterns (Whitfield 1979). It also leads to several

testable predictions about cortical activity evoked by odors and the resulting sensory perceptions. Although there is great evolutionary conservation of peripheral features of odor processing across phyla (Hildebrand and Shepherd 1997), mammals have invested a substantial metabolic commitment to paleo- and neocortical olfactory circuits. This chapter will review the structure and function of the olfactory cortex, and describe data on the associative, multimodal, state- and expectation-dependent nature of cortical odor processing. This chapter will also attempt to outline issues that need to be addressed before we can answer how the olfactory cortex contributes to odor perception (Figure 14.1).

## 14.2 THE OLFACTORY CORTEX

The olfactory cortex is typically defined as those areas receiving direct input from the olfactory bulb. This includes wide regions of the olfactory peduncle and ventrolateral forebrain in rodents, and more ventromedial regions in humans. Specific target structures include the anterior olfactory nucleus, the olfactory tubercle, the cortical nucleus of the amygdala, the piriform cortex, and even lateral regions of the entorhinal cortex, though direct input to the entorhinal cortex from the olfactory bulb is minor. Beyond these primary olfactory cortical regions, neocortical areas with substantial olfactory input (e.g., via the primary olfactory cortex) include the lateral entorhinal cortex and the orbitofrontal cortex. This chapter will focus primarily on the piriform cortex and the orbitofrontal cortex, given that the majority of recent work on odor-evoked cortical activity has emphasized these regions (though see, anterior olfactory nucleus: [Lei et al. 2006; Yan et al. 2008]; olfactory tubercle: [Zelano et al. 2007]).

In addition to olfactory bulb input, the olfactory cortex has strong, often reciprocal relationships with limbic areas, such as the amygdala (Majak et al. 2004), the hypothalamus (Price et al. 1991), and the perirhinal cortex (Luskin and Price 1983). There is also heavy innervation by modulatory inputs from the horizontal limb of the diagonal band of Broca (acetylcholine), the raphe nucleus (serotonin), and the locus coeruleus (norepinephrine) (Shipley and Ennis 1996). Together, these limbic and modulatory connections allow behavioral state, hedonic valence, arousal, and attention to shape cortical responses to odor.



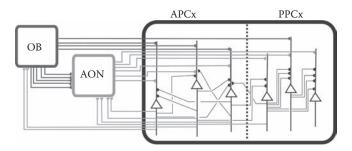
**FIGURE 14.1** Illustration of the major connections with the piriform cortex. In addition to afferent input from the olfactory bulb and anterior olfactory nucleus, the piriform cortex receives input from neuromodulatory as well as higher order processing centers. These connections allow behavioral state, memory, and emotional state to alter responses in the piriform cortex. It should also be noted that the bidirectional connections allow the piriform cortex to alter higher order processes. Abbreviations: OB = olfactory bulb; AON = anterior olfactory nucleus; PCX = piriform cortex; ENT = entorhinal cortex; OFC = orbitofrontal cortex; AMG = amygdala; DMN = dorsomedial nucleus of the thalamus; HDB = horizontal limb of the diagonal band; LC = locus coeruleus; RN = raphe nucleus; ACh = acetylcholine; NE = norepinephrine; 5-HT = serotonin.

# 14.3 AUTOASSOCIATIVE CIRCUITS WITHIN THE PRIMARY OLFACTORY CORTEX

As opposed to primary sensory regions of the neocortex (somatosensory, visual, and auditory), there is no apparent spatial organization of afferent projections or sensory-evoked activity within the olfactory cortex. In the olfactory bulb, olfactory sensory neurons expressing the same olfactory receptor distributed across the olfactory epithelium converge onto a small number of individual glomeruli. Given that different receptors impart different ligand-binding characteristics to the sensory neurons (Malnic et al. 1999; Araneda et al. 2000, see also Chapter 7), the homogenous sensory neuron convergence to different glomeruli creates odor-specific spatial patterns of activity within the olfactory bulb (Stewart et al. 1979; Rubin and Katz 1999; Wachowiak et al. 2000; Johnson and Leon 2007, see also Chapters 12 and 13). The associated second-order neurons and local interneurons appear to form columns aligned with the glomeruli (Guthrie et al. 1993; Willhite et al. 2006). Thus, different spatial patterns of glomeruli and their associated mitral cells are activated in response to different odorants. Furthermore, there may be spatial organization within these patterns, with for example, glomeruli and associated neurons tuned to aldehydes clustering together, while those tuned to alcohols clustering together in a different region of the bulb (Imamura et al. 1992; Johnson and Leon 2007). Similarly, within the primary sensory neocortex, neurons within a given cortical column display similar tuning characteristics to stimulus features such as auditory wavelength, orientation of visual stimuli, or location of touch on the body surface. Columns near to each other tend to contain neurons expressing similar, though not identical receptive fields (Figure 14.2).

In both the olfactory bulb and the sensory neocortex, these organized tuning characteristics emerge through precise patterns of afferent input from sensory receptors. In the sensory neocortex, these highly organized patterns of activity form topographical maps that are preserved as information is transmitted through the thalamus to the neocortex. Opportunities for interaction between neocortical columns exist through lateral and association connections, which allow for higher order feature detection. Similarly, olfactory sensory receptor neurons converge onto specific olfactory glomeruli; however, this topographic map does not appear to be conserved beyond the olfactory bulb. The basic spatial organization seen in most sensory systems is absent in the piriform cortex.

Afferents to the piriform cortex and the hippocampus are organized entirely differently (Neville and Haberly 2004). In contrast to the tight spatial patterning of sensory neuron input to the olfactory bulb and mitral cells, mitral cell projections to the piriform cortex terminate in broad patches



**FIGURE 14.2** A highly simplified illustration of network connections between PCX, OB, and AON. The blue lines represent forward projecting axons. Green lines represent feedback connections. The intracortical association fibers are shown in black. The association fibers in APCx project both rostrally and caudally, whereas in PPCx they tend to project caudally. These network connections, as well as others, suggest different processing functions in APCx vs PPCx.

(Ojima et al. 1984; Buonviso et al. 1991). There may be some regional difference in termination between anterior and posterior subregions, but there does not appear to be any clear topographic pattern of input from the spatially organized olfactory bulb to the piriform cortex. These broad afferent patches allow for extensive overlap and convergence of input from different mitral cells conveying information from different olfactory sensory receptor neurons.

Furthermore, in addition to the convergence afforded by afferent fiber overlap, there is an extensive excitatory association fiber system within the piriform cortex. This system is recurrent and autoassociative. That is, individual pyramidal cells receiving input from a specific pattern of mitral cells can feedback onto themselves and their neighbors (which may receive a different random combination of afferent inputs) to enhance convergence and potential associations between different patterns of mitral cell input. A single pyramidal cell may terminate on over one thousand other pyramidal cells in widely disparate regions of the piriform cortex (Johnson et al. 2000). Importantly, the association fiber system expresses activity-dependent associative synaptic plasticity (Kanter and Haberly 1990; Poo and Isaacson 2007; Stripling and Galupo 2008). Thus, as particular input patterns (odors) become familiar, the association fiber system records them through changes in synaptic weight. As discussed below, this allows the system to complete degraded or noisy patterns, which allows perceptual stability (Hasselmo et al. 1990; Granger and Lynch 1991; Hopfield 1991; Haberly 2001), and is a classic characteristic of autoassociative networks.

## 14.4 ODOR-EVOKED ACTIVITY IN THE PRIMARY OLFACTORY CORTEX

Based on the structure of the piriform cortex, it does not appear to be purely a primary sensory area, but a multimodal association cortex. As a result, there are predictions that can be made. First, activity evoked by a particular odorant should be distributed across the piriform cortex, given the distributed afferent input and the widespread associational connections. Second, there should be minimal spatial organization of odor-response patterns of individual neurons, with, for example, neighboring cells potentially responding to very different odorants depending on the specific set of afferent and association connections on those individual neurons. Third, mixtures of odorants should be processed more synthetically and distinct from their components in the piriform cortex than in the olfactory bulb, based on the extensive convergence within the cortex and the columnar organization and limited afferent convergence within the olfactory bulb. Fourth, experience should strongly influence odor processing within the cortex, given the hypothesized role of association fiber plasticity in cortical circuit function. Fifth, piriform cortical activity should reflect not only odor stimulation, but also odor associations, given the extensive reciprocal connections with limbic and neocortical areas. Each of these predictions is supported by the following findings.

#### 14.4.1 GLOBAL SPATIAL PATTERNS

Data from <sup>14</sup>C-2-deoxyglucose metabolic imaging (Cattarelli et al. 1988), c-fos and other immediateearly gene mapping (Illig and Haberly 2003; Zou and Buck 2006), voltage-sensitive dye mapping (Litaudon et al. 1997), and ensemble unit recording (Rennaker et al. 2007), all show no evidence of strong spatial topography in odor-evoked or olfactory bulb-evoked activity within the piriform cortex. There may be regional variations in sensitivity to given odorants (Illig and Haberly 2003; Zou and Buck 2006), for example, between the dorsal and ventral regions of the anterior piriform cortex (Illig and Haberly 2003). There are also differences between the anterior and posterior piriform cortex, with the posterior piriform cortex neurons, having lower spontaneous activity, being more selective (narrowly tuned) to unfamiliar odors than the anterior piriform cortex neurons in anesthetized rats (Litaudon et al. 2003). However, as predicted from the distributed patterns of afferent and intracortical association fibers, the precise odor-specific spatial patterning evident in the olfactory bulb is largely lost in the piriform cortex.

#### 14.4.2 LOCAL SPATIAL PATTERNS

The loss of global odor-specific spatial activity patterns in the piriform cortex is also evident at the single neuron level. Single-unit pairs recorded in the anterior piriform cortex with a single electrode, and thus assumed to be near neighbors, showed differences in both spontaneous and odor-evoked activity. Thus, for example, spontaneous activity of neighboring neurons was poorly correlated across the respiratory cycle, with neurons often completely out of phase (Rennaker et al. 2007). Furthermore, neighboring neurons display different odor tuning, with one of the pair responsive to a particular odor and the other not (Rennaker et al. 2007). Again, this is consistent with a highly distributed afferent input amplified by a highly distributed intracortical association fiber system.

#### 14.4.3 Odor Mixture Processing

Olfactory cortical neurons appear to respond to mixtures differently than olfactory bulb mitral cells, in accord with the convergence of multiple inputs to cortical neurons. However, the difference can be subtle. For example, a mitral cell may respond to a variety of odor mixtures, as long as they include a component that activates olfactory sensory neurons modulating that cell's activity. Similarly, cortical neurons may also respond to multiple mixtures either because of the strength of single afferent inputs (Franks and Isaacson 2006), or because of activity in specific combinations of afferent and association fiber inputs to that cell. Furthermore, responses to odor mixtures can be affected at all levels of the olfactory pathway by ligand interactions at the olfactory receptor, local circuit effects within the olfactory bulb, and larger circuit interactions within olfactory cortical areas. For example, mixture suppression effects have been observed at all levels of the olfactory pathway where it has been looked for (Derby et al. 1991; Kadohisa and Wilson 2006b). Thus, comparison of mitral cell and piriform cortical responses to novel, random odor mixtures may not tell the whole story about how the cortex responds to mixtures (see below).

Nonetheless, mixture responses in two olfactory cortical areas, the anterior olfactory nucleus (Lei et al. 2006) and the piriform cortex (Wilson 2000b; Barnes et al. 2008), have been compared to responses to the same odors by mitral cells. Mitral cells responding to a mixture of molecularly dissimilar components generally respond to only a single or small number of the components, while olfactory cortical neurons may respond to many of the components (Lei et al. 2006). This fits with the idea that mitral cells respond to a mixture due to the presence of a particular component, while cortical neurons respond to a convergence of multiple components. In fact, a subset of cortical neurons may require convergent input from different afferent populations in order to be activated (Lei et al. 2006; Zou and Buck 2006). Furthermore, cross-adaptation studies of familiar binary mixtures and their molecularly dissimilar components (Wilson 2000a). In contrast, anterior piriform cortex neurons showed minimal cross-adaptation between familiar mixtures and their components (Wilson 2000a). These results suggest that cortical neurons treat mixtures as distinct objects, different from their components.

Recordings of cortical single-unit ensembles further demonstrate that cortical circuits allow completion of degraded input patterns evoked by complex odor mixtures. As noted above, pattern completion is a defining feature of autoassociative networks. Most natural odors are combinations of many odorants. While each may contribute differentially to the overall mixture quality, there can be natural variation in the presence or strength of individual components, yet the percept remains stable. For example, the aroma of a chardonnay wine may include dozens of individual components, each detectable when presented alone. However, when presented together, their individual qualities are lost perceptually to allow a single percept of chardonnay (Jinks and Laing 2001). In many cases, the complete percept can be recreated, even if some of the individual components are missing, i.e., the olfactory system can fill in the missing gaps and complete the chardonnay pattern. In contrast, inclusion of a single abnormal component, such as mold on a cork, may completely

alter the chardonnay percept—even if the abnormal component cannot be explicitly identified. Autoassociative networks are ideal for solving such pattern completion and pattern separation (discrimination) problems.

Complex odor mixtures evoke complex spatiotemporal patterns in the olfactory bulb glomerular layer and mitral cell output (Lin et al. 2006). Even very subtle changes in the sensory input, e.g., loss of a component within the mixture, can be detected by mitral cell ensembles and thus are reflected in olfactory bulb output patterns (Barnes et al. 2008). However, piriform cortex single-unit ensembles fail to decorrelate these very subtle losses from their response to the complete mixture, thus allowing a completion of the full pattern from the degraded pattern (Barnes et al. 2008). This pattern completion should result in difficulty in behavioral discrimination of the complete mixture from its degraded version, and it does (Barnes et al. 2008). However, continued degradation of the mixture with loss of additional components rapidly produces a pattern completion process in piriform cortical ensembles, enhancing decorrelation of the complete mixture from its morphed version. This enhanced pattern separation corresponds to excellent behavioral discrimination. Similarly, inclusion of a single abnormal component within a complex mixture (similar to the cork taint in wine), leads to marked cortical decorrelation, pattern separation, and behavioral discrimination. (Barnes et al. 2008).

#### **14.4.4 ODOR EXPERIENCE EFFECTS**

Cortical responses to odors are highly dynamic, reflecting past experience over both short and long time courses. This means that cortical odor responses not only reflect sensory neuron input, but also past experience and previous odor associations.

The piriform cortex rapidly adapts to stable odor input in rats (Wilson 1998), mice (Kadohisa and Wilson 2006b), and humans (Sobel et al. 2000). The cortical adaptation occurs despite relatively stable responses of olfactory bulb mitral cells (Wilson, 1998). In rats, short-term cortical odor adaptation is induced by metabotropic glutamate receptor-mediated synaptic depression of mitral cell input to the cortex (Best and Wilson 2004). Blockade of these receptors prevents both cortical adaptation (Best and Wilson 2004) and habituation of simple odor-evoked behaviors (Best et al. 2005; McNamara et al. 2008). Increases in noradrenergic input to the piriform cortex, as might occur with an increase in arousal or vigilance, can induce dishabituation and return of odor-evoked responses (Smith et al. submitted). The cortical odor adaptation is highly odor-specific, especially to familiar odors (Wilson 2003). This odor specificity allows the piriform cortex to use adaptation to segment new odors from an odorous background (Kadohisa and Wilson 2006b; Linster et al. 2007).

Associative conditioning also modifies cortical odor responses (Litaudon et al. 1997; Zinyuk et al. 2001; Moriceau and Sullivan 2004; Li et al. 2008). In general, learned familiar odors evoke enhanced activation of the piriform cortex as measured with c-fos immunohistochemistry or metabolic activity (Moriceau and Sullivan, 2004; Li et al. 2008), though specific effects may differ between anterior and posterior subregions (Litaudon et al. 2003; Kadohisa and Wilson, 2006a). At the global (fMRI; Li et al. 2008) and cortical ensemble (Kadohisa and Wilson 2006a) levels, associative conditioning also enhances decorrelation of encoding between similar odors within the anterior piriform cortex. In the posterior piriform cortex, which has been implicated in encoding higher order odor quality or category (e.g., "fruity"; Gottfried et al. 2006), associative conditioning can lead to a decrease in decorrelation of similar odors or odors experienced within mixtures (Kadohisa and Wilson 2006a). These changes in the posterior piriform cortex may underlie the observed merging of odor quality perceptions of odors experienced in binary mixtures (Stevenson 2001).

Some of this cortical modification reflects changes that occur as early as the olfactory bulb (Sullivan and Leon 1986; Fletcher and Wilson 2003; Martin et al. 2004b; Harley et al. 2006; Mandairon et al. 2006; Doucette and Restrepo 2008), although odor learning also modifies synaptic physiology and cellular biophysics within the piriform cortex itself (Roman et al. 1993; Saar et al. 2002; Saar and Barkai 2003; Cohen et al. 2008). Nonetheless, these findings suggest that

odor processing is linked very early in the sensory pathway to odor associations and hedonics. The piriform cortex is strongly, reciprocally linked to the amygdala and orbitofrontal cortex, thus, odor "meaning" extracted by those regions may feedback to the piriform cortex and shape processing (see below).

Recent work in the gustatory cortex suggest that single-unit ensembles within this primary sensory cortex go through several stages reflecting different network states encoding not only stimulus identity, but also hedonic valence or palatability (Jones et al. 2007). The ensemble encoding is also modulated by behavioral state or attention (Fontanini and Katz 2006). The changes in network activity may reflect not only local circuit interactions, but also larger scale interactions between the gustatory cortex and other areas such as the amygdala (Grossman et al. 2008). These kinds of single-unit ensemble analyses need to be applied to the olfactory cortex in the future.

#### 14.4.5 DESCENDING CONTROL AND MULTIMODAL CONVERGENCE

Odor responses in the primary olfactory cortex reflect not only olfactory sensory neuron activity, but also behavioral state, context, and current and past associations with the odor. Information regarding these diverse nonolfactory features comes from descending inputs from neocortical and limbic areas, as well as modulatory inputs from the basal forebrain and brainstem.

In recordings from awake animals performing odor-guide behaviors, piriform cortical activity reflects not only odor stimulus quality, but also other aspects of the animal's behavior, such as approach to the odor sampling port, movement from the sample port to the reward port, and consummation of the reward (Schoenbaum and Eichenbaum 1995; Zinyuk et al. 2001). This nonolfactory activity may reflect the fact that even olfactory bulb neurons respond to multiple aspects of the behavioral task (Kay and Laurent 1999; Rinberg and Gelperin 2006), and the broader circuit context within which the piriform cortical activity rests.

For example, both cholinergic inputs from the basal forebrain horizontal nucleus of the diagonal band of Broca (Linster et al. 1999) and noradrenergic input from the brainstem nucleus locus coeruleus (Bouret and Sara 2002) modulate spontaneous and evoked piriform cortical activity. For example, activation of the locus coeruleus enhances entrainment of piriform cortical single-unit spontaneous activity to the respiratory cycle, and enhances (primarily) odor-evoked activity (Bouret and Sara 2002). Given that locus coeruleus activity is affected by novel or intense stimuli (such as unconditioned stimuli) and behavioral state (Sara et al. 1994), odors temporally associated with these conditions should impinge on a hyperexcitable piriform cortex, enhancing the probability that the odor input will be memorized by cortical circuits (Linster and Hasselmo 2001). Interestingly, as noted above, norepinephrine can also induce dishabituation of habituated odor responses (Smith et al. submitted), further enhancing the probability of learning odors associated with significant, nonolfactory events.

These same modulatory inputs may also be important for decreasing sensory-evoked activity within the piriform cortex during down states. During slow-wave sleeplike states in urethaneanesthetized rats, piriform cortical single-units become less responsive to the olfactory bulb and odor input (Fontanini and Bower 2005; Murakami et al. 2005). This state-dependent gating of sensory throughput is similar to the role the thalamus plays in thalamocortical sensory systems.

In addition to changes in intrinsic piriform cortical activity, state, context, and task demands can also affect coupling of cortical activity to that in other regions. Such changes in coupling, usually measured with coherence of local field potential oscillations, can reflect the varying strength of functional connectivity between local or distant brain regions. For example, beta frequency oscillations in local field potentials (around 15–40 Hz, though this varies between laboratories) generally reflect the information flow between two regions at some distance, for example, between the olfactory bulb and piriform cortex, or between the hippocampus and olfactory bulb. Activity can be recorded in different brain regions within these frequency ranges, and under specific conditions, the activity in the different regions can become entrained, or coherent. This increase in coherence

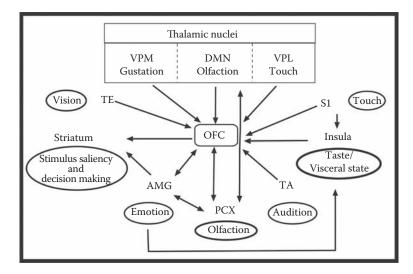
suggests an increased functional coupling between areas, and a potential enhancement in transfer or linking of information.

During odor conditioning or in response to a biologically significant odor, beta frequency oscillations are enhanced in several discrete olfactory regions, such as the olfactory bulb (Ravel et al. 2003), the piriform cortex (Martin et al. 2004a), and the entorhinal cortex (Chabaud et al. 2000). However, in addition to the increase in these oscillations in specific areas, there is also an increase in coherence within beta frequency between, for example, the hippocampus and olfactory bulb (Martin et al. 2007). Beta frequency oscillations may also be indicative of feedback input to the olfactory bulb from the entorhinal cortex, perhaps enhancing identification or recognition of learned odor features (Kay et al. 1996). Thus, as odors gain significance, or even have their significance modulated by behavioral state (e.g., hunger; Chabaud et al. 2000), large-scale circuits begin to act coherently, linking odor representations to their meaning, and to expectations or context.

## 14.5. BEYOND THE PRIMARY OLFACTORY CORTEX

Odorant stimulation evokes activity in a variety of regions beyond the primary olfactory cortex. In humans, many neocortical areas are activated by odor stimulation, with the specific contribution of individual areas influenced by the route of odor stimulation (ortho- or retronasal; Small et al. 2005), stimulus intensity (Bensafi et al. 2008), stimulus hedonics (Bensafi et al. 2007; Grabenhorst et al. 2007), attention (Zelano et al. 2005; Plailly et al. 2008), expectation and/or multimodal context (Gottfried and Dolan 2003), stimulus familiarity (Plailly et al. 2005), and imagery of odors (Djordjevic et al. 2005). As described below, in many cases, functional imaging in humans has led the way in mapping these larger circuit olfactory processes, though important observations in animals add to the overall understanding (Figure 14.3).

Of particular note as an olfactory processing area is the neocortical orbitofrontal cortex. The dorsomedial nucleus of the thalamus projection to the orbitofrontal cortex is the olfactory thalamocortical pathway most comparable to other sensory systems. However, the olfactory orbitofrontal cortex receives odor input not only from the thalamus, but also directly from the piriform



**FIGURE 14.3** Illustration of the major connections with the orbital frontal cortex. The multimodal sensory input, along with connections to emotional areas, memory, and higher order processing, suggests a complex modulation of olfactory responses in the piriform cortex related to behavioral, emotional, and past experience. It is likely that many of these connections are bidirectional. Abbreviations: VPL = ventral posterolateral nucleus; VPM = ventral posteromedial nucleus; DMN = dorsomedial nucleus of the thalamus; TE = inferior temporal visual cortex; TA = superior temporal auditory association cortex.

cortex (Johnson et al. 2000). This piriform cortex-orbitofrontal cortex connection is reciprocal (Illig 2005), allowing descending neocortical control over piriform cortex activity. Furthermore, the orbitofrontal cortex is highly multimodal, with single neurons responsive to olfactory, gustatory, somatosensory, and visual stimuli (Rolls 2001, 2004). Finally, in addition to multimodal convergence, activity within the orbitofrontal cortex reflects affective response to, or incentive value of, odors (Schoenbaum et al. 2003a), and is strongly modified by past odor associations (Rolls et al. 1996; Schoenbaum et al. 1999) and current motivational state (Critchley and Rolls, 1996b; O'Doherty et al. 2000).

As expected in an olfactory region, single-units in the orbitofrontal cortex can respond to odor stimulation, and can discriminate between different odors, potentially having more narrow odor receptive fields than mitral cells (Tanabe et al. 1975). As in the piriform cortex, there does not appear to be any detectable spatial topography in odor-evoked activity within the orbitofrontal cortex, though this has not been closely examined. In primates, there is a lateralization in odor-evoked activity, with the right orbitofrontal cortex showing the dominant response (Zatorre et al. 1992).

As mentioned above, orbitofrontal cortex activity reflects odor-reward associations in rats (Schoenbaum et al. 2003b; van Duuren et al. 2007, 2008), humans (Gottfried et al. 2002), and nonhuman primates (Critchley and Rolls 1996a). Thus, for example, in a task wherein different odors predicted different sized rewards, single-units and single-unit ensembles responded differentially to the predicted reward size as signaled by the learned odors (van Duuren et al. 2007, 2008). Such associative learning is also correlated with the enhanced strength of orbitofrontal synaptic projections to the anterior piriform cortex (Cohen et al. 2008), providing a learning-induced top-down modulation of piriform odor processing.

As with piriform cortex activity described above, recent work has examined orbitofrontal cortex functional connectivity with other components of large-scale brain networks. Again, these analyses examine not only odor-evoked activity within a specific brain region, but also how that activity is correlated with or entrained to activity in other brain regions. For example, as noted above, the two primary sources of odor information to the orbitofrontal cortex come from the piriform cortex and the dorsomedial nucleus of the thalamus. However, both the dorsomedial nucleus of the thalamus (Amaral et al. 2003) and the piriform cortex (Majak et al. 2004) receive input from the basolateral amygdala, a region critical for emotional memory and hedonic reactions (LeDoux 2003).

Thus, a large-scale network exists involving (at least) the orbitofrontal cortex, the piriform cortex, the amygdala, and the dorsomedial nucleus of the thalamus. Functional connectivity between several of these circuit nodes has been examined during odor learning and attention. For example, activity within both the basolateral amygdala and orbitofrontal cortex increase during reward expectation in an odor-learning task (Schoenbaum et al. 1998). Lesions of the amygdala reduce these reward-based responses in the orbitofrontal cortex, leaving cortical responses more restricted to odor quality coding alone (Schoenbaum et al. 2003b). This suggests a potential convergence within the orbitofrontal cortex of odor information driven from the piriform cortex and perhaps the dorsomedial nucleus of the thalamus, and hedonic or value information from the amygdala via the dorsomedial nucleus of the thalamus.

Furthermore, attention plays an important role in functional connectivity within this circuit. For example, attention to odors enhances the functional connectivity between the dorsomedial nucleus and the orbitofrontal cortex in humans, compared to attention to tones (Plailly et al. 2008). There was no effect of attention on piriform cortex functional connectivity to the orbitofrontal cortex (Plailly et al. 2008). In addition, selective attention to odor pleasantness enhances activation of the orbitofrontal cortex relative to conditions where attention was directed to odor intensity (Rolls et al. 2008). Given that this hedonic information appears to be derived from the amygdala-dorsomedial nucleus-orbitofrontal cortex pathway, both studies suggest an attentional modulation of thalamic input to the orbitofrontal cortex. A similar role for the thalamus in attention has been described in other sensory systems (McAlonan et al. 2008).

Finally, in addition to the orbitofrontal cortex, odor-evoked activity has been described in the entorhinal cortex in rats (Kay, 2005; Petrulis et al. 2005) and humans (Cerf-Ducastel and Murphy, 2003; Bensafi et al. 2008), and the cingulate cortex (Grabenhorst et al. 2007). Entorhinal cortex damage in Alzheimer disease is associated with impaired odor identification (Wilson et al. 2007). Cingulate cortical activity may reflect hedonic valence, especially negative valence of odors (Grabenhorst et al. 2007). Further mapping of odor-evoked neocortical activity is warranted.

#### 14.6 SUMMARY

The neuroanatomy of the olfactory bulb-piriform cortex circuit is highly conserved in vertebrates, and thus might be thought to play a basic, critical role in odor perception. The piriform cortex neural architecture is that of an autoassociative array, and it seems to serve in a pattern recognition capacity to deal with complex spatiotemporal patterns of olfactory bulb output in response to complex natural odors. Plasticity of intrinsic intracortical connections permits memorization of familiar patterns, which promotes both completion of slightly degraded patterns to allow perceptual stability, and separation/decorrelation of more distinct patterns to allow perceptual discrimination. Given the diverse limbic and neocortical inputs to the piriform cortex, the odor representations can also include, perhaps inextricably, nonolfactory components such as learned associations. Thus, odor-evoked activity within the piriform cortex is spatially diffuse (nontopographic) and modulated by behavioral state, expectations, and past experience.

The piriform cortex, in turn, projects both directly and indirectly to the orbitofrontal cortex. In addition to olfaction, the orbitofrontal cortex receives multimodal sensory inputs. Through network interactions with the piriform cortex, the thalamus, and the amygdala (among other areas), the orbitofrontal cortex appears to encode learned or intrinsic value together with odor quality. Thus, learned or state-dependent changes in hedonic valence or value of the odor can affect odor-evoked activity within the orbitofrontal cortex.

Based on these and other findings, it is the cortex that drives what is commonly experienced as our conscious sense of smell. While olfactory sensory neuron activity places constraints on odor perception, it is cortical processing that allows the perception of synthetic odor objects, reactions of pleasure or disgust, and memories of home.

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# 15 Memory and Plasticity in the Olfactory System: From Infancy to Adulthood

Anne-Marie Mouly and Regina Sullivan

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One of the most characteristic features of odor memory in humans is the rather unique ability of odors to vividly trigger the evocation of emotional experiences. This property might be sustained by the direct connections established by the olfactory bulb and piriform/olfactory cortex on two structures involved in emotion and memory, namely the amygdala and hippocampus. In animals, memory for environmental odors plays a vital role because it regulates many behaviors that are crucial for survival. Highly emotional or at least particularly ethologically relevant olfactory learning occur during an animal's major life events. Moreover, conditioning procedures can be used to induce emotional olfactory learning, thus allowing an experimental approach in a laboratory environment. The aim of the present chapter is to illustrate some aspects of the neurobiology of odor emotional memory in rats, both in infancy and adulthood. We focus on fear conditioning at both developmental points, since it constitutes one of the most adapted and classical paradigms

to study emotional memory in animals and has been the focus of intense investigation. We begin by reviewing the available literature describing the neural circuits involved in odor fear conditioning in adult rats, and conclude the chapter by reviewing the ontogeny of odor fear conditioning. The same experimental paradigm can be supported by overlapping but distinct neural circuits, and lead to dramatically different behavioral outcome, depending on the age at learning. This approach highlights how the fear system changes to meet the ecological demands of different life stages.

## **15.1 INTRODUCTION**

Although olfaction is generally assumed to play a minor role in humans compared to vision or audition, it is also commonly accepted that odors have a rather unique status for eliciting memories. Indeed, one of the most striking features of odor memory in humans resides in the amazing power of odors to vividly trigger the evocation of autobiographical experiences. This property has been referred to as the "Proust phenomenon" by some authors (Chu and Downes 2000, 2002) because of the well-known literary anecdote reported by Proust (1919) at the beginning of his novel, *Swann's Way*, wherein the flavor of a madeleine cake dipped into a cup of tea unwillingly caused the remembrance of an old detailed memory.

"The memory suddenly appears before my mind. The taste was that of the little piece of madeleine which on Sunday mornings at Combray (because on those mornings I did not go out before churchtime) my aunt Léonie used to give to me, dipping it first in her own cup of real or lime-flower tea', leading him to the conclusion that 'When from a long-distant past nothing subsists ... the smell and taste of things remain poised for a long time ... and bear unfaltering, in the tiny and almost impalpable drop of their essence, the vast structure of recollection."

A number of psychological studies have experimentally investigated the so-called Proust phenomenon in human subjects. Different works have shown that odor-cued memories are more emotional than memories triggered by visual or verbal cues (Chu and Downes 2000; Herz and Cupchik 1995; Herz 1998; Herz and Schooler 2002; Willander and Larsson 2007). Odor-cued memories have also been described as more vivid than memories evoked by corresponding words (Chu and Downes 2002). In addition, the feeling of being brought back in time to the occurrence of the event is experienced as stronger for odor-cued memories than memories evoked by words and pictures (Herz and Schooler 2002; Willander and Larsson 2006). Interestingly, it was also shown that autobiographical memories evoked by olfactory information were older than memories associated with verbal information (Chu and Downes 2000; Willander and Larsson 2006). Specifically, most odor-cued memories were located to the first decade of life (<10 years), whereas memories associated with verbal and visual cues peaked in early adulthood (11-20 years). This observation is in agreement with the fact that olfaction emerges very early in ontogeny and with research showing that associative odor learning begins very early in life (Schaal et al. 2000; Van Toller and Kendal-Reed 1995). Taken together, these studies suggest that human olfaction is unique in its ability to cue the emotional aspects of autobiographical memory, including experiences formed early in life.

In animals, memory for environmental odors plays a vital role because it regulates many behaviors that are crucial for their survival. Highly emotional or at least particularly ethologically relevant olfactory learning occurs during an animal's major life events. For instance, and as will be developed in Section 15.3, during the early postnatal (PN) period when altricial pups are crucially dependent on their mother for feeding and maternal care, unusually rapid and strong olfactory learning occurs. The newly learned odor becomes preferred and is approached to bring the infant in contact with the mother, although the odor also controls the complex sequencing of motor patterns to support nipple attachment in rats (Cheslock et al. 2000; Hofer and Sullivan 2001; Pedersen et al. 1982) and rabbits (Distel and Hudson 1985; Coureaud et al. 2006; Schaal et al. 2003). At adulthood, two types of powerful ethological olfactory learning have been described in the literature. The first occurs in the postpartum period, when the mother learns the odor of the newborn and is critical for the mother to accept and nurse the young. This has been eloquently described in the postpartum sheep (Levy et al. 2004; Nowak et al. 2007; Keller et al. 2004, 2005; Brennan and Kendrick 2006) and rat (Fleming et al. 1999; Levy et al. 2004), and is the first step in the establishment of mother–young bonding. The second example of ecologically significant odor learning involves chemosensory individuality (Brennan 2004) and mate recognition (Brennan and Keverne 1997), which regulate reproductive behavior in mice (see also Chapter 6). Thus, particularly robust and rapid odor learning occurs throughout the life span during life transitions critical for survival and reproductive success.

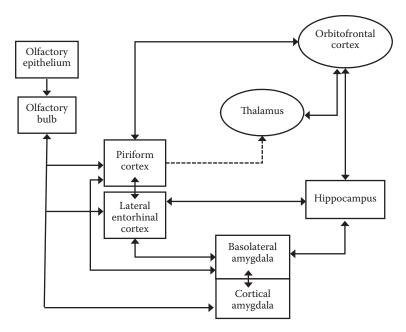
Rapid, emotional learning is also necessary during threatening situations, to avoid that danger in the future. This type of learning is modeled by fear conditioning, where a temporal association of a neutral stimulus (i.e., tone or odor) is made with a noxious stimulus (i.e., shock) after only a few pairings. The formerly neutral stimulus acquires the ability to elicit defensive or escape responses critical in avoiding the danger and is dependent upon plasticity in the basolateral complex of the amygdala (LeDoux 2000; Fanselow and Gale 2003; Davis and Whalen 2001; Maren 2003; Otto et al. 2000). This paradigm has permitted enormous progress in our understanding of emotional learning in a controlled laboratory environment. More recently, this approach has been extended to humans with remarkable convergence in the underlying neural circuitry during acquisition, expression, and extinction, suggesting some homologous control of emotional learning in rats and humans (LaBar et al. 1998).

The aim of the present chapter is not to present an exhaustive review of the neurobiology of these different olfactory learning tasks, but rather to concentrate on those inducing emotional olfactory memory in rats, both at early PN ages and at adulthood. The vast majority of the existing studies on fear conditioning have been carried out using auditory or visual modalities. However, odors are at least as potent as auditory or visual cues in sustaining learned fear. Therefore, we specifically review the literature describing the neural circuit involved in odor fear conditioning in adult and infant rats, but we begin by presenting a brief review of the olfactory pathways within the brain.

#### 15.1.1 NEUROANATOMY OF THE OLFACTORY PATHWAYS

Olfactory sensory neurons lie in the olfactory epithelium, which covers the back of the nasal cavity. The axons of the olfactory sensory neurons travel through the cribriform plate to the olfactory bulb, which is the first relay of olfactory information (Figure 15.1). The olfactory bulb output neurons send direct projections to the olfactory cortex, including the anterior olfactory nucleus, the olfactory tubercle, and the piriform or olfactory cortex (Shipley and Ennis 1996; Haberly and Price 1977; Carmichael et al. 1994). The first unusual characteristic of the mammalian olfactory pathways is that, unlike other sensory systems, it does not need to pass through the thalamus to reach cortical areas. Indeed, there is no thalamic relay between the first relay of sensory information, namely the olfactory bulb, and the primary olfactory cortex, as is the case for other sensory modalities (Figure 15.1). Similarly, there is no obligatory thalamic relay for olfactory information to reach the orbitofrontal cortex. Indeed, although the piriform cortex sends sparse projections to the mediodorsal thalamus, thereby disynaptically targeting the orbitofrontal cortex, it establishes direct monosynaptic connections with the orbitofrontal cortex (Datiche and Cattarelli 1996; Tanabe et al. 1975; Yarita et al. 1980; Ray and Price 1992; Carmichael et al. 1994). These observations suggest that the thalamus might be less relevant for olfaction than for the other senses (Shepherd 2005; Wilson and Stevenson 2006), but see Plailly et al. (2008) for an alternative view.

Another unique feature of the olfactory pathways is the olfactory bulb's output neurons rapid connections to structures crucially implicated in emotion and memory, namely the amygdala and the



**FIGURE 15.1** Schematic representation of the main components of the olfactory pathways. Diagram illustrates the intimate link between the olfactory bulb and the limbic areas (amygdala and hippocampus).

hippocampus. Indeed, the main olfactory bulb makes dense monosynaptic contacts with nuclei of the corticomedial amygdaloid group, including the nucleus of the lateral olfactory tract, the cortical nucleus of the amygdala, and the periamygdaloid cortex (Price 1973; McDonald 1998). These observations led Swanson and Petrovich (1998) to suggest that the corticomedial amygdala is an integral component of the olfactory system. These superficial nuclei are a major source of the projections from the amygdala to the hypothalamus (Price et al. 1991). In contrast, the deeper amygdaloid nuclei, including the basolateral nuclear group, do not receive projections from the olfactory bulb and receive relatively weak projections from the olfactory piriform cortex (Krettek and Price 1978; Luskin and Price 1983; Ottersen 1982). However, they receive fairly dense projections from the corticomedial nuclei within the amygdala (Savander et al. 1996). Taken together, these anatomical data suggest that compared to the other sensory modalities, olfactory information has a unique direct access to the amygdala.

In addition, both anatomical (Price 1973; Haberly and Price 1977) and electrophysiological (Biella and de Curtis 2000; Wilson and Steward 1978; Boeijinga and Van Groen 1984; Mouly et al. 1998, 2001; Mouly and Di Scala 2006; Biella et al. 2003) studies have shown that the olfactory bulb and the piriform cortex also send direct projections to the lateral entorhinal cortex via the lateral olfactory tract. The lateral entorhinal cortex, in turn, has been shown to project to the hippocampus via the lateral perforant path (Witter and Amaral 1991). Furthermore, in addition to providing a gateway to the hippocampus, the lateral entorhinal cortex are the basolateral amygdala and the cortical nuclear complex (Brothers and Finch 1985; McDonald and Mascagni 1997). The lateral entorhinal cortex is, therefore, in a position to transmit information from olfactory cortical areas to two major telencephalic components, the amygdala and the hippocampus, and back to olfactory areas through reciprocal connections (Swanson and Kohler 1986; Insausti et al. 1997).

## 15.1.2 ONTOGENESIS OF THE OLFACTORY PATHWAYS

The olfactory system is functional at birth and, as outlined in Section 15.3, odors support behaviors critical for pups' survival at birth. Amazingly, pups smell, discriminate, and process odors, yet the

olfactory circuit of pups is quite immature compared to the adult rat brain. While the olfactory bulb is fairly mature at birth and functional odor maps in the glomerular layer are well defined, considerable development continues throughout the preweanling period (Guthrie and Gall 1995, 2003). For example, gamma-aminobutyric acid (GABA) interneurons, presumably critical for odor discrimination in adults (Ennis et al. 1998), are sparse, yet pups show odor discrimination (Fletcher et al. 2005). Additionally, centrifugal input from the rest of the brain begins to enter the bulb prenatally, although substantial input continues through weaning. For example, norepinephrine (NE) fibers from the locus coeruleus (LC) are present at birth, although NE released by these terminals greatly decreases as pups mature (McLean and Shipley 1991), while terminal numbers increase. While minor cholinergic (ACh) projection neurons arrive in the bulb as early as the first day of life to innervate the modified glomerular complex, most fibers begin to reach the bulb at PN day 6 and start to show the more typical homogenous distribution across the bulb layers (McLean and Shipley 1991; Le Jeune and Jourdan 1991). At birth, there is also a transient large number of neurons expressing acetylcholinesterase (AChE), although this begins to decrease around PN15, and shows low adult levels by PN30 (Le Jeune and Jourdan 1991). In the next relay station for olfactory information, the olfactory cortex, considerable development also occurs during the first two weeks of life and later (Brunjes et al. 2005; Schwob and Price 1984; Walz et al. 2006). However, the functional significance of the developing piriform on pup behavior has remained elusive, primarily due to lack of research attention.

The amygdala also continues to develop during the period. The basolateral complex first emerges at E17, with most neurons produced between E20–PN7. Other amygdaloid nuclei lag behind the basolateral by a few days. The amygdala continues to develop until adolescence (Morys et al. 1998, 1999; Berdel and Morys 2000; Dziewiatkowski et al. 1998; Berdel et al. 1997). Olfactory information is received by the amygdala in the early neonatal period (Schwob and Price 1984) and the infant amygdala responds to odors (Thompson et al. 2008). However, the sequential emergence of amygdala-dependent behaviors throughout the preweanling period suggests important intra-amygdala connections continue (Hunt et al. 1994). Functional maturation of the amygdala and its role in pups' behavior is outlined in Section 15.3.

Connectivity between olfactory structures continues to become more complex. For example, in the infant rat, frontal cortex development and its projections to the piriform cortex and amygdala are also protracted, with anatomical maturation and connections occurring between PN8–14, although "adult-like" laminar cellular organization occurs as pups complete the second week of life (Bouwmeester et al. 2002; Verwer et al. 1996; Kolb et al. 1996). Together, the protracted development of these brain areas suggests that the neonate has a unique circuitry for odor processing that underlies developmental changes in emotional learning.

#### 15.2 EMOTIONAL ODOR MEMORY IN ADULT RATS

#### 15.2.1 FEAR CONDITIONING: A TOOL FOR STUDYING EMOTIONAL MEMORY IN ANIMALS

Among the few emotions that can be detected and quantified in animals, fear is indubitably the most common. When a rat encounters a threatening stimulus (e.g., the odor of a predator), it expresses fear reactions, including physiological (blood pressure increase, stress hormones release, hypoalgesia, etc.) and behavioral responses (Takahashi et al. 2005; Maren and Quirk 2004; Dielenberg and McGregor 2001). Among these, fear-potentiated startle and fear-induced freezing (defined as complete immobility) are universal fear responses seen in many animal species, including humans. These innate fear responses to a natural threat can be classically conditioned to an experimental neutral stimulus and this is referred to as fear conditioning (LeDoux 2000; Maren 2001; Myers and Davis 2007; Maren and Quirk 2004). Fear conditioning consists of pairing an initially neutral stimulus (the conditioned stimulus or CS) with an aversive unconditioned stimulus (US), generally a mild foot-shock. After a few trials, re-exposure to the CS alone elicits fear responses, such as

freezing behavior, assumed to be part of an anticipatory response to threat and danger (Rosen and Schulkin 1998).

The vast majority of studies devoted to investigate the neural basis of fear conditioning have used auditory and, to a lesser extent, visual cues as conditioned stimuli. In particular, the neural pathways involved in auditory fear conditioning have been well characterized (LeDoux 2000; Maren 2001; Maren and Quirk 2004; Myers and Davis 2007). The information carried by the auditory CS can take one of two pathways: either directly from the thalamus to quickly reach the amygdala or the CS can travel from the auditory thalamus to the auditory cortex before reaching the amygdala. These thalamic and cortical areas send -projections to the lateral nucleus of the amygdala, which is a site of CS-US convergence. The lateral nucleus, in turn, projects to the central amygdala, which controls the expression of fear responses by way of projections to brainstem areas. The major conclusion of these studies is that the amygdala plays a critical role in linking external stimuli to defense responses through synaptic plasticity. The amygdala would, therefore, be essential to the formation and storage of fear memories as far as auditory or visual stimuli are used for conditioning. Extinction of fear memory would involve an inhibitory action exerted by the prefrontal cortex onto the amygdala network (Milad and Quirk 2002), although disruption of memory within the amygdala has also been implicated (Davis et al. 2003). In addition, the hippocampus seems to play an important role in regulating the context dependence of fear memory after extinction.

Intriguingly, whereas olfaction plays a dominant role in rat's behavior from the very first hours of life throughout adulthood and, as outlined above, olfactory areas establish particularly direct connections with the amygdala, very few studies have used odor cues as CS in fear conditioning paradigms.

#### 15.2.2 NEUROBIOLOGY OF OLFACTORY FEAR CONDITIONING IN RATS

Behavioral researches have shown that olfactory fear conditioning induces robust emotional responses. Otto et al. (1997, 2000), measuring freezing behavior as an index of learned fear, reported that olfactory fear conditioning resulted in robust and long-lasting associations between an odor and a foot-shock. Richardson et al. (1999) and later, Paschall and Davis (2002), using fear-potentiated startle as another measure of learned fear, showed that an odor previously paired with shock was a particularly effective stimulus for potentiating the startle response in rats. Moreover, in a subsequent study, Richardson et al. (2002) reported that extinction of learned fear potentiation of startle occurs more slowly with an olfactory CS than with auditory or visual cues. Thus, odors are at least as efficient as other types of sensory stimuli to induce learned fear responses.

## 15.2.2.1 The Role of the Amygdala

Concerning the neural basis of olfactory fear conditioning, there is a consensus in the existing literature that the amygdala plays a crucial role. Indeed, Cousens and Otto (1998) first showed that pre- and posttraining excitotoxic lesions of the basolateral amygdala abolished the acquisition and expression of olfactory fear conditioning in rats. In another study, Walker et al. (2005) evaluated the respective contribution of the basolateral and medial amygdala to olfactory fear conditioning by infusing antagonists of glutamate receptors into these areas prior to either odor-shock pairings or retention test using fear-potentiated startle. Pretraining blockade of N-methyl D-aspartate (NMDA) receptors into the basolateral amygdala disrupted fear conditioning to the odor, while pretest blockade of  $\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)/kainate receptors disrupted fear-potentiated startle to the learned odor. Neither compound blocked fear conditioning when infused into the medial amygdala prior to training, but pretest AMPA/kainate receptors antagonist infusion did block fear-potentiated startle. Using an electrophysiological approach, Rosenkranz and Grace (2002) performed in vivo intracellular recordings in the lateral nucleus of the basolateral

amygdala during acquisition of an odor fear conditioning under anaesthesia, in rats. Their data revealed that repeated pairing of an odor with foot-shock induced enhanced postsynaptic potential responses in neurons of the basolateral amygdala, and that this modification resulted from local changes in synaptic efficacy, and was dependent on dopamine.

Another set of studies has investigated more specifically the involvement of the amygdala in the consolidation of odor fear conditioning. Specifically, Killpatrick and Cahill (2003) used reversible inactivation of the basolateral amygdala with tetrodotoxin following paired odor-shock presentation in rats. This treatment resulted in a deficit in learning, thus suggesting that the basolateral amygdala plays a role in consolidation of odor fear conditioning. Similarly, Rattiner et al. (2004) measured the expression of brain-derived neurotrophic factor (BDNF) mRNA in the amygdala after olfactory as well as visual fear conditioning. BDNF is known to play a critical role in activity-dependent synaptic plasticity and has been implicated as mediator of hippocampal-dependent learning and memory (Hall et al. 2000; Kesslak et al. 1998). The authors showed that BDNF expression was increased in the basolateral amygdala two hours after fear conditioning and that this increase occurs independentlearning of the Sensory modality of the CS.

Taken together, these data suggest that the basolateral amygdala plays a major role in the acquisition, consolidation, and retention of olfactory fear conditioning, thus extending to odor cues the previous observations obtained with auditory and visual CS.

#### 15.2.2.2 The Role of Extra-Amygdala Structures

Beside the above studies specifically addressing the role of the basolateral amygdala, sparse studies have investigated the potential involvement of other target structures in this learning. Among them, Ressler et al. (2002) used genes identified in a kainic acid model of synaptic plasticity, as in situ hybridization probes during the consolidation period after odor fear conditioning. They found that following odor-shock pairings, these genes (such as immediate early genes like c-Fos and Zif 268) were transcriptionally regulated in several brain areas, including the basolateral and medial amygdala, the piriform, perirhinal, and insular cortices, along with the endopiriform nucleus and the habenula. These data suggest that plasticity after odor fear conditioning may depend on a broad neural circuit that includes the amygdala.

Other studies have investigated more precisely the role of extra-amygdala areas in odor fear conditioning. Laviolette et al. (2005) examined the possible role of the medial prefrontal cortex (mPFC) in the acquisition and encoding of odor fear learning at the behavioral and single-neuron level. A subpopulation of neurons in the mPFC that received monosynaptic inputs from the basolateral amygdala demonstrated strong associative responding to odors paired previously with foot-shock by increasing spontaneous activity and bursting activity. In addition, systemic or intra-mPFC blockade of dopamine receptors prevented this emotional associative learning in neurons of the mPFC and blocked the expression of olfactory conditioned fear. These results demonstrate that mPFC neurons that receive a functional input from the basolateral amygdala actively encode emotional learning and that this process is under the dependence of dopamine.

In another study using aspirative or excitotoxic lesions, Herzog and Otto (1997, 1998) investigated the role of the perirhinal cortex. They reported that rats with perirhinal cortex lesions presented a robust attenuation of fear conditioning to olfactory stimulus, thus, suggesting that the perirhinal cortex is an important component of the neural circuit supporting the association between olfactory cues and foot-shock. Later, Schettino and Otto (2001), measuring c-Fos expression related to the acquisition and expression of olfactory fear conditioning, reported that the anterior region of the medial nucleus of the amygdala, as well as the ventral perirhinal cortex, could be critically involved in this learning. Using the same technique, Funk and Amir (2000) also showed that presentation of the aversive conditioned odor stimulus induced an enhanced increase in levels of Fos expression in the main and accessory olfactory bulbs and in the anterior olfactory nucleus, as well as in the infralimbic and orbital cortices.

#### 15.2.2.2.1 The Role of the Hippocampus

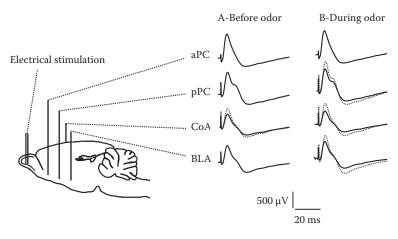
In the literature, there is widespread agreement that the hippocampus is not critically involved in the acquisition of discrete CS-US associations (Anagnostaras et al. 2001; Lopez-Fernandez et al. 2007; Maren et al. 1997; Phillips and LeDoux 1992; Schafe et al. 2001), and this also stands for odor-shock pairings (Otto and Poon 2006). Interestingly however, when the odor is presented as a contextual cue instead of an elemental discrete cue, the hippocampus seems to be engaged in the learning of the association. Specifically, Parsons and Otto (2008) examined the effects of temporary inactivation of the dorsal hippocampus on fear behavior in an explicitly nonspatial contextual fear conditioning paradigm, in which olfactory stimuli served as temporally and spatially diffuse contexts. They found that the transient lesion of the dorsal hippocampus produced both anterograde and retrograde deficits in olfactory contextual conditioning, while sparing the acquisition and expression of freezing to a discrete auditory or olfactory CS. These data suggest that the dorsal hippocampus participates in both the acquisition and retention of unimodal olfactory contextual fear conditioning.

## 15.2.2.2.2 The Role of the Piriform Cortex

Among the other candidate structures for participating in the neural network sustaining odor fear learning, the piriform cortex has been the core of particular attention. The piriform cortex is the largest of the olfactory areas and is usually divided into anterior and posterior parts. Olfactory bulb output neurons synapse primarily on pyramidal cells in the anterior piriform cortex and only send lighter, more distributed inputs to the posterior piriform cortex (de Olmos et al. 1978; Haberly and Price 1978; Haberly 2001). Furthermore, the ratio of associative to afferent input is higher in more posterior regions of the piriform cortex (Haberly and Price 1978; Luskin and Price 1983; Datiche et al. 1996). Moreover, whereas the anterior piriform cortex receives input from the orbitofrontal cortex (Datiche and Cattarelli 1996; Illig 2005), the posterior piriform cortex receives afferents from the basolateral amygdala (Johnson et al. 2000; Majak et al. 2004). In addition, electrophysiological studies have further emphasized this dissociation. Indeed, Litaudon and Cattarelli (1996), using optical recording of the whole piriform cortex activity, showed that following repetitive electrical stimulation of the olfactory bulb, a higher degree of inhibition was observed in the ventromedial anterior part of the piriform cortex than in the posterior part. Using the same technique, Litaudon and Cattarelli (1995) also found that the amplitude of a late component of the evoked activity was larger in the posterior than in the anterior piriform cortex, a result which was confirmed by Mouly et al. (1998) on classical evoked field potential signals. In the domain of synaptic plasticity, Jung et al. (1990) showed that the induction of long-term potentiation in vitro was easier in posterior piriform cortex slices than in the anterior slices. Taken together, the anatomical and functional differences observed between anterior and posterior piriform cortices suggest that these two parts of the olfactory cortex could play differential roles in odor fear conditioning.

Jones et al. (2007) examined BDNF mRNA expression across the olfactory system following fear conditioning in mice. They found a specific increase in BDNF mRNA in animals receiving paired foot-shocks in the posterior piriform cortex and the basolateral amygdala. This was in contrast to the unpaired and odor-alone treatments, where BDNF mRNA was increased in the olfactory bulb and the anterior piriform cortex only, but not the higher olfactory areas. Thus, BDNF mRNA in the olfactory bulb and the anterior piriform cortex seems to be induced simply by exposure to olfactory stimuli, regardless of whether the odor is associated with the shock. In contrast, BDNF mRNA is only induced in the posterior piriform cortex and the basolateral amygdala when there is an association between odor and shock to support learning.

These data complement previous observations reported in our group. Indeed, using an electrophysiological approach, we investigated whether olfactory fear conditioning induces synaptic changes within the olfactory pathways, mainly focusing on olfactory cortical areas, namely the piriform cortex and the amygdala cortical and basolateral nuclei (Sevelinges et al. 2004). For this,



**FIGURE 15.2** Evoked field potentials were induced in the anterior piriform cortex (aPC), the posterior piriform cortex (pPC), the cortical (CoA) and basolateral nucleus of the amygdala (BLA) in response to stimulation of the olfactory bulb, either before (plain line) or 24 h after (dashed line) acquisition of odor fear conditioning. The signals were collected before odor presentation and in the presence of the odor. Learning was accompanied by a lasting increase in signal amplitude in CoA before odor introduction, and a transient facilitation in BLA, CoA, and pPC during odor presentation.

evoked field potential signals induced in behaving animals by electrical stimulation of the olfactory bulb were collected simultaneously at the level of the anterior piriform cortex, the posterior piriform cortex, the cortical nucleus of the amygdala, and the basolateral nucleus of the amygdala. Recordings were made before learning and during a retention test carried out 24 h after acquisition of odor fear conditioning. The data showed that learning was accompanied by a lasting increase in signal amplitude in the cortical amygdala, i.e., an increase observed before CS presentation (Figure 15.2A). In addition, introduction of the learned CS odor induced a transient facilitation in the basolateral amygdala, as well as in the cortical amygdala and the posterior piriform cortex (Figure 15.2B). These data suggest that the posterior piriform cortex and the amygdala are key structures in the neural circuit underlying odor fear conditioning.

In a recent study (Hégoburu et al. In preparation), we used high temporal resolution (1 min sampling rate) intracerebral microdialysis, in order to study the accurate temporal dynamic of neurotransmitter release in these two structures during the course of learning acquisition. Simultaneous monitoring of GABA and glutamate was performed in both the basolateral amygdala and the posterior piriform cortex, during the odor fear acquisition session including six odor-shock pairings. A transient increase in glutamate was observed in the amygdala for the two first CS–US pairings, whereas a significant increase in GABA levels was shown for the second pairing only. In contrast, in the posterior piriform cortex, transient increases in both GABA and glutamate levels were found after each odor-shock pairing. Interestingly, for both neurotransmitters, the increase observed in the amygdala occurred earlier in time (1-2 min) than the increase in the posterior piriform cortex. High temporal resolution microdialysis, therefore, allowed us to show a temporal dynamic of activation of these two structures during the course of successive pairings, with the involvement of the amygdala during the first odor-shock associations preceding neurotransmitter release in the posterior piriform cortex, after which the latter alone supports pairing-induced modifications. This led us to suggest that the amygdala has a crucial, but temporally limited, role in signaling changes during a potentially dangerous situation, while the posterior piriform cortex may contribute to higher mnemonic processes, including storage of the detailed attributes of the learned stimulus

Taken together, these data bring further support to the growing body of evidence showing that the anterior and posterior piriform cortices play different complementary roles in memory processes. Specifically, the anterior piriform cortex would be mainly involved in coding the sensory features of the odor (Roesch et al. 2007), and in simple forms of short-term memory like habituation (Kadohisa and Wilson 2006; Wilson 1998, 2000) or perceptual learning (Barnes et al. 2008; Wilson and Stevenson 2003), whereas the posterior piriform cortex would be involved in the learning and recall of associations between odorants and information from other sensory modalities (Chabaud et al. 1999, 2000; Mouly et al. 2001; Mouly and Gervais 2002; Kadohisa and Wilson 2006; Haberly 2001; Litaudon et al. 2003; Sevelinges et al. 2004, 2008; Calu et al. 2007; Martin et al. 2004).

## 15.2.3 Odor Fear Conditioning in Humans

During the past ten years, several studies using the functional magnetic resonance imaging (fMRI) technique, have investigated the networks involved in fear conditioning in humans (Cheng et al. 2003; Buchel and Dolan 2000; LaBar et al. 1998). Most of these studies suggest that the amygdala is part of the circuit, thus corroborating the data from the animal literature. Interestingly, in a recent work, Li et al. (2008) used an odor fear conditioning paradigm in humans in order to investigate how aversive learning enhances perceptual acuity of sensory signal. During conditioning, the CS odor presentation coterminated with electric shock (US), whereas presentation of its chiral counterpart (enantiomer) was not associated with the US. The authors combined multivariate fMRI with olfactory psychophysics, and hereby showed that initially indistinguishable odor enantiomers become discriminable after aversive conditioning. In parallel, fMRI data demonstrated progressive decreases in amygdala activity evoked by the learned aversive odor as learning proceeded, together with increases in the orbitofrontal cortex. Interestingly, the authors also measured changes in activity in the piriform cortex. They reported that spatial patterns of fMRI activity in the posterior piriform cortex between the two enantiomers were highly correlated before conditioning, but became more distinct after conditioning. This effect was specific to the posterior piriform cortex, as it was not observed in the anterior piriform cortex. These findings confirm and extend previous data reported by the same group, showing a double dissociation in the piriform cortex, whereby posterior regions encode quality, and anterior regions encode structure (Gottfried et al. 2006; Li et al. 2006). Taken together, these data indicate that aversive learning induces plasticity in the posterior piriform cortex that correlates with gains in odor enantiomer discrimination. This led the authors to propose that fear conditioning has the capacity to update perceptual representation of predictive cues, in addition to its well-recognized role in the acquisition of conditioned responses.

The data obtained in human odor fear conditioning are strikingly similar to those described in rats and suggest that whereas the amygdala plays a crucial role, a broad network of structures is involved in the learning, among which the piriform cortex seems to endorse a privileged status.

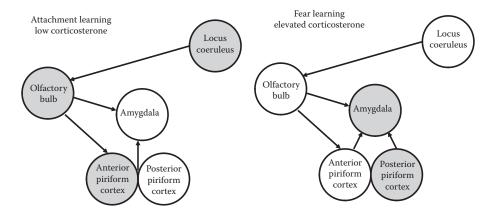
# 15.3 DEVELOPMENT OF ODOR LEARNING MEMORY

The importance of emotional olfactory learning is evident in the rat pups' first learning experience, when pups must learn the mother's odor to attach to their caregiver. However, pup learning is strikingly different from adult learning and appears to accommodate pups' unique environmental demands. Pups are born immature and completely dependent upon their mother for survival, yet become independent only three weeks later. During this time, the rat pups' world repeatedly changes as they transition from intrauterine life, to nest life, and finally to independence. As will be illustrated in this chapter, different forms of learning emerge, disappear, and sometimes coexist as pups mature. Here, we will review the relationship between a few forms of olfactory learning that change as the demands and importance of attachment learning to the mother and more "adultlike" learning interplay. We will review three types of infant learning: (1) attachment learning that involves learning the maternal odor and expressing the complex behaviors controlled by that odor; (2) olfactory fear learning that emerges as pups begin to venture outside the nest; and (3) malaise learning that enables pups to avoid odors paired with gastrointestinal distress, learning that occurs throughout development but with a changing neural circuit.

#### 15.3.1 Attachment Learning and Maternal Odor

Perhaps the most dramatic transition occurs at birth when infant rats face the daunting cognitive task of transitioning from intrauterine life to nest life. Not only do pups need to learn their mother's odor within minutes of birth, but also they must rapidly express this learning with approach to their mother and nipple attachment for their first nursing bout. While the mother does assist pups in maintaining contact with her, and prenatal learning supports the PN learning, the PN learning and expression are essential (Pedersen and Blass 1982; Polan and Hofer 1999). If pups do not learn the maternal odor or their olfactory function is disrupted, they have little chance of survival (Singh et al. 1976). Furthermore, since the maternal odor is dependent on the maternal diet, pups must continue to learn the maternal odor throughout the preweanling period.

The maternal odor guides pups to the mother and controls social interactions with the mother, including nipple attachment. During the first week and a half of life, this attachment learning shows very rapid and robust acquisition, as well as some intriguing unique characteristics similar to imprinting. This sensitive period for pups' rapid learning appears well adapted for the unique demands of life within the nest and the transition to independence that occurs a few weeks later. For most of the sensitive period, the motorically immature pups remain in the nest nursing. However, the mother entering and leaving the nest is sometimes associated with distress to pups when the mother drags still-attached pups out of the nest and steps on others. Thus, pups' exposure to the maternal odor occurs during nursing and grooming by the mother, but with occasional painful stimuli from the mother. Importantly, pups feel pain (Fitzgerald 2005; Collier and Bolles 1980; Barr 1995; Emerich et al. 1985; Shair et al. 1997; Stehouwer and Campbell 1978). As illustrated in Figure 15.3, one of the most strikingly unique learning characteristics of pups, is the ability of both painful stimuli (0.5 mA shock, tailpinch) and presumably pleasant stimuli (milk, warmth, stroking that mimics maternal grooming) to both support learning of approach responses and nipple attachment. Specifically, pairing a novel odor (CS) with either a painful or presumably pleasant reward, results in pups showing subsequent approach responses to that odor. Indeed, this learning occurs within the nest with a mother nursing her pups, but also when pain is induced by a stressed mother (Gilles et al. 1996), who exhibits rough handling and transporting of pups without nursing (Roth and Sullivan 2005). This attenuated odor-aversion or fear learning during pups' early life is not the only learning restriction for pups, since inhibitory conditioning and passive avoidance are also



**FIGURE 15.3** Y-maze test in sensitive-period PN8 pups using the CS odor or the maternal odor vs clean home cage bedding (response to bedding not shown). Infant rat pups can be classically conditioned to approach a novel odor with previous pairings with either painful stimuli (i.e., 0.5 mA shock) or pleasant stimuli (i.e., stroking). Pups approach responses to the learned attachment odors are as robust as that exhibited to natural maternal odor. Mere experience with a novel odor or unpaired presentations of the novel odor and reward does not support learning.

attenuated (Blozovski and Cudennec 1980; Collier and Mast 1979). Finally, these early life learned odors are not just approached as a preferred odor, but appear to take on characteristics of maternal odor and also support nipple attachment when the natural maternal odor is removed (Pedersen and Blass 1982; Raineki et al. in progress; Hofer et al. 1976). Thus, this learning is unique both in its expression and acquisition and has been characterized as mammalian imprinting (Hofer and Sullivan 2001).

The early life odor learning that supports acquisition of the maternal odor is also potentiated by other unique learning features in infancy. Specifically, exposure to the odor CS before classical conditioning (latent inhibition) and uncorrelated presentations of the CS and reward (learned irrelevance), both of which retard/inhibit learning in adults, either enhance or have no effect on the young infant rat's learning (Stanton 2000; Stanton et al. 1998; Campbell and Spear 1972; Hoffmann and Spear 1988; Rescorla 1967, 1988; Rush et al. 2001; Siegel and Domjan 1971; Spear and Rudy 1991). Finally, while sequential presentation of the CS and the reward optimizes learning in adults, simultaneous presentation of stimuli enhances associations in young pups (Barr et al. 2003; Cheslock et al. 2003).

Thus, rat pups have myriad unique learning characteristics that potentiate the odor learning required for pups to maintain proximity to their mother. While one normally thinks of early life learning as providing enhancement of learning, such as in imprinting, limitations on aversive learning in early life are actually widespread. For example, shocking a chick while it is following the surrogate caregiver during imprinting, enhances following of the surrogate caregiver. Just hours after the imprinting critical period ends, a similar shocking procedure causes the chick to avoid the surrogate (Salzen 1967). An analogous situation has been demonstrated in young dogs (Stanley 1962). Indeed, mistreating a puppy with shock or rough handling by a human caregiver results in a strong attachment to that caregiver. A similar phenomenon has been repeatedly documented in nonhuman primate colonies, where the young of abusive mothers still form and maintain strong attachments to their caregiver (Harlow and Harlow 1965; Maestripieri et al. 1999; Sanchez et al. 2001; Suomi 2003; Nemeroff 2004). Finally, clinical and epidemiological studies on children indicate that "abuse" results in children forming and maintaining strong attachments to their caregiver (Bowlby 1965; Helfer et al. 1997). Indeed, the wide phylogenetic representation of tolerance of pain during attachment learning and its maintenance suggests a phylogenetically preserved system for attachment (Hofer and Sullivan 2008).

## 15.3.2 THE NEUROBIOLOGY OF ATTACHMENT ODOR LEARNING: OLFACTORY BULB, ANTERIOR PIRIFORM CORTEX, AND LOCUS COERULEUS (LC)

During the sensitive period, the neural circuitry supporting the attachment olfactory learning and memory also appears unique and is associated with enhanced odor-induced olfactory responding. Similarly to the behavior, the neural responses evoked by natural maternal odors and attachment odors learned within the nest or in controlled learning experiments appear similar (Harley et al. 2006; Sullivan et al. 1990; McLean and Shipley 1991; Woo et al. 1996; Roth and Sullivan 2005; Shionoya et al. 2006; Leon and Johnson 2003; Yuan et al. 2000; Pedersen and Blass 1982; Smotherman 1982). This modified olfactory bulb response has been documented using 2-DG, c-Fos, intrinsic optical imaging, and modified single-unit response patterns of the bulb's output neurons, mitral/tufted cells (Johnson et al. 1995; Sullivan and Leon 1986; McLean and Shipley 1991; Wilson et al. 1987; Woo et al. 1987). Recent work also suggests that this early life odor preference learning supporting attachment is also encoded in the anterior piriform cortex (Moriceau and Sullivan 2006; Roth and Sullivan 2005). Due to limited research on the immature piriform cortex, its function in pup learning remains unknown. As reviewed above, both the anterior and posterior piriform cortices have an important yet different role in adult odor learning (Sevelinges et al. 2004; Kadohisa and Wilson 2006; Calu et al. 2007; Brosh et al. 2006; Brennan and Kendrick 2006; Martin et al. 2004, 2006; Bernabeu et al. 2006; Datiche et al. 2001).

During the sensitive period, the olfactory attachment odor and the olfactory bulb learninginduced changes are dependent upon high levels of NE (Sullivan and Wilson 1994; Harley et al. 2006; Sullivan et al. 2000), with the LC as the bulb's sole source of NE (McLean and Shipley 1991). The sensitive-period LC is uniquely responsive to diverse types of sensory stimulation (e.g., 1 s stroking, 0.5 mA shock), which may account for the wide range of sensory stimuli to support infant learning. Additionally, the sensitive-period LC releases abundant NE (Rangel and Leon 1995; Moriceau et al. In preparation) due to its prolonged response (20–30 s), which is in sharp contrast to the very brief millisecond response found in older pups and adults (for a review, see Nakamura and Sakaguchi 1990). Finally, the infant LC fails to habituate with repeated stimulus presentations, while the adult LC rapidly habituates with only two or three presentations (Nakamura and Sakaguchi 1990; Nakamura et al. 1988; Sara et al. 1994).

With maturation (>PN10), NE release from the LC is greatly reduced and no longer sufficient to produce odor preference learning in postsensitive-period pups (Sullivan and Wilson 1994; Moriceau and Sullivan 2004; Sullivan et al. 2000; Harley et al. 2006). This changing developmental role for LC and its reduced NE release is due to the functional emergence of the LC's inhibitory  $\alpha$ 2 noradrenergic autoreceptors that quickly terminate the LC's response and greatly reduces NE release (Nakamura and Sakaguchi 1990; McGaugh 2006). Interestingly, the olfactory bulb's ability to support odor attachment learning can be reinstated by direct infusion of high levels of NE or by blocking the LC's  $\alpha$ 2 noradrenergic autoreceptors to reinstate the LC's prolonged response and abundant NE release (Moriceau and Sullivan 2004). With this postsensitive-period LC maturation, NE begins to play a more modulatory role of enhancing or attenuating memories in a manner similar to adults (for reviews, see McGaugh 2006; Ferry and McGaugh 2000). Thus, while many neurotransmitters have a role in early olfactory learning in neonatal rats, NE appears to have a particularly important role.

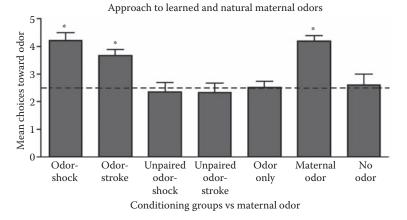
#### 15.3.3 Emergence of Olfactory Fear Learning

At around PN10, crawling transition to walking, and the probability of pups leaving the nest increases. Pup odor attachment learning is not appropriate for outside the nest, where learning needs to reflect the consequences of pain or pleasure to ensure pups survival in preparation for independent life. Indeed, the sensitive-period attachment learning ends at this time, at least when pups are outside the nest. Furthermore, new learning emerges and pups begin to exhibit olfactory fear conditioning from odor-pain pairings (0.5 mA shock, tailpinch), with pups avoiding the odor in a two-odor choice Y-maze and freezing to CS odor presentation in a glass jar (Roth and Sullivan 2005; Haroutunian and Campbell 1979; Moriceau and Sullivan 2006; Moriceau et al. 2006; Sullivan and Leon 1986; Sullivan et al. 2000) (Figure 15.4).

#### 15.3.4 THE NEURAL BASIS OF FEAR CONDITIONING THE PIRIFORM CORTEX AND AMYGDALA

The amygdala is required for fear conditioning (Blair et al. 2001; Sananes and Campbell 1989; Fanselow and LeDoux 1999; Davis et al. 1997; Fanselow and Gale 2003; Herzog and Otto 1997; Maren 2003; Pape and Stork 2003; Pare et al. 2004; Rosenkranz and Grace 2002; Schettino and Otto 2001; Sevelinges et al. 2004; Sigurdsson et al. 2007; Davis et al. 2003). Indeed, as fear conditioning emerges in pups, the basolateral complex of the amygdala shows learning-associated changes, as indicated by c-Fos and 2-DG (Moriceau and Sullivan 2006; Moriceau et al. 2006; Sullivan et al. 2000). Importantly, a causal relationship has been established between the currently emerging fear conditioning and the amygdala, since temporarily silencing the amygdala with muscimol abolishes pups fear conditioning (Moriceau and Sullivan 2006).

Input to the amygdala also changes at this age. Before the emergence of the amygdala-dependent fear conditioning, the olfactory bulb and anterior piriform were associated with the odor-shock induced odor preference (Moriceau et al. 2006; Roth and Sullivan 2005). The olfactory bulb



**FIGURE 15.4** The graphic on the left illustrates the neural basis of the attachment learning that occurs when an odor is paired with myriad stimuli (stroking, warmth, milk, 0.5 mA shock). Without the mother present, this learning occurs in pups younger than PN10. However, the sensitive period of learning can be extended to PN15 if the odor-shock conditioning takes place in the mother's presence or other situations that are associated with low CORT levels (adrenalectomy, CORT receptor blockade in the amygdala). The graphic on the right illustrates the neural basis of fear conditioning in postsensitive-period pups or older pups with heightened CORT levels (chronic stress, CORT receptor agonists within the amygdala), which occurs when a novel odor is paired with 0.5 mA shock. The olfactory bulb, anterior piriform cortex, and LC constitute the attachment neural circuit, while the fear conditioning neural circuit activates the posterior piriform cortex and the amygdala during fear conditioning in older pups.

appears to indicate an odor is important with the hedonic value coded by the anterior piriform cortex (Moriceau et al. 2006; Roth and Sullivan 2005). With the emergence of fear conditioning, this same procedure only produces learning-associated changes in the posterior piriform cortex, which has a well-documented role in learning in adults. While the role of the posterior piriform is not yet understood in pups, it does have highly distributed reciprocal interactions with the prefrontal, amygdaloid, entorhinal, and perirhinal cortices (Johnson et al. 2000), which suggests a major role in information synthesis (Wilson and Stevenson 2006; Haberly 2001).

## 15.3.5 CORTICOSTERONE (CORT) INCREASES INDUCE THE PREMATURE EMERGENCE OF FEAR CONDITIONING

Perhaps the most convincing evidence that amygdala maturity does not account for pups emerging ability to learn fear is our ability to pharmacologically induce amygdala-dependent fear conditioning in pups as young as PN5 (Roth and Sullivan 2005; Moriceau and Sullivan 2004, 2006; Moriceau et al. 2006; Wiedenmayer and Barr 2001). Specifically, the-sensitive-period attachment learning required low corticosterone (CORT) during conditioning. Pups have a stress hyporesponsive period (SHRP) when stressors, such as shock, fail to induce a CORT increase (Grino et al. 1994; Levine 1962, 1967, 2001; Rosenfeld et al. 1992; Guillet and Michaelson 1978; Butte et al. 1973; Guillet et al. 1980; Cate and Yasumura 1975; Walker et al. 1986; Henning 1978). At around PN10, pups have a sufficient stress-induced CORT release to support fear conditioning (Moriceau et al. 2006). However, since maternal presence blocks shock-induced CORT release in these older pups, pups will continue to learn to prefer odors paired with shock if the mother is present during conditioning (Stanton et al. 1987; Stanton and Levine 1990; Suchecki et al. 1993; Moriceau and Sullivan 2006). In fact, these older pups can rapidly switch between the amygdala-dependent fear learning and attachment learning during odor-shock conditioning, dependent upon CORT levels modified by the mother (Moriceau and Sullivan 2006). While the mother's ability to block stress-induced CORT release may appear unusual, it is fairly widespread in mammals. For example, maternal presence in adolescent guinea pigs, the presence of the mate in voles, the presence of peers in nonhuman primates, social affiliation in humans all reduce/block stress-induced CORT release (DeVries et al. 2003; Hennessy et al. 1995). With maturation, CORT takes on a more modulatory role of modifying the learned aversion and inhibitory conditioning (Corodimas et al. 1994; Hui et al. 2004; Pugh et al. 1997; Roozendaal et al. 1996, 2002; Thompson et al. 2004). Since early life stress prematurely increases pups CORT, early life stress prematurely terminates attachment learning and facilitates the precocious emergence of fear conditioning, which has recently been demonstrated in our laboratory (Moriceau et al. In preparation).

#### 15.3.6 DEVELOPMENT OF MALAISE OLFACTORY LEARNING

The developmental delay in pups' emotional learning and amygdala-dependent fear conditioning is not due to pups' inability to learn an odor avoidance. Indeed, young pups can learn to avoid odors, although malaise and introceptive distress rather than exteroceptive pain is responsible for this learning (Haroutunian and Campbell 1979). Specifically, rat pups can learn to avoid odors paired with malaise throughout ontogeny, including before birth (Shionoya et al. 2006; Smotherman 1982; Miller et al. 1990; Rudy and Cheatle 1983; Coopersmith and Leon 1986; Hoffmann et al. 1987). We have recently assessed the neurobiology of malaise learning. Since this learning occurs during the prenatal period, which is prior to the maturation of brain structures critical for adult odor-malaise learning (i.e., amygdala) (Touzani and Sclafani 2005; Berdel and Morys 2000; Berdel et al. 1997; Morys et al. 1998), we searched for a unique circuit for learning. We used two means of inducing malaise in pups, odor-LiCl and 1 mA high shock (Haroutunian and Campbell 1979), both of which also produce gastrointestinal distress in pups (Davenport 1950; Stern et al. 1969; Raineki et al. 2008). In sharp contrast to the important role of the amygdala in adult odor-malaise learning, young pups use the olfactory bulb and the piriform cortex to support learning (Shionoya et al. 2006). Indeed, it is not until pups are close to weaning that the amygdala is used in odor-malaise learning, which is almost a week later than the emergence of amygdala-dependent fear conditioning (Sullivan et al. 2000; Roth and Sullivan 2005; Moriceau and Sullivan 2006; Moriceau et al. 2006). Thus, despite the similar appearance in the odor-aversion learning produced by odor-0.5 mA fear conditioning and the odor-malaise learning, the neural circuitry supporting this learning differs, indicating caution is required when assuming that similar appearing behaviors in development use the same neural structures.

Maternal presence also modifies pups odor-malaise learning. Specifically, if pups are nursing while conditioned with odor-LiCl pairings, pups fail to learn the aversion and learn to prefer the odor (Shionoya et al. 2006). These data complement pups conditioning with taste-LiCl, which is blocked if pups are nursing (Martin and Alberts 1979; Gubernick and Alberts 1984; Melcer et al. 1985; Kehoe and Blass 1986).

As illustrated in Table 15.1, pups show remarkable odor learning abilities, which are uniquely adapted to the pups' ecological demands as the transition from complete dependence changes to complete independence. Indeed, pups transition from unique neural circuits supporting their unique learning to circuits consistent with that documented in the adult. More remarkable is the dramatic effect of maternal presence on pup learning, which provides pups to "switch" to attachment learning in the mother's presence. It is important to note that the similarity in behavior between fear and malaise learning (odor avoidance) suggests that similar behaviors across development may be an unreliable tool to assess brain development. Indeed, the age at which the amygdala supports pups' emotional learning from odor-shock conditioning differs from the age the amygdala supports malaise learning, suggesting caution when extrapolating functional brain development from one behavior to another. Additionally, while this odor-0.5 mA shock conditioning begins to produce fear conditioning at PN10, fear conditioning is blocked and the early life odor preference learning is extended via suppression of the amygdala or if the mother is present (Moriceau and Sullivan 2006). A similar learning constraint exists with odor-LiCl and taste-LiCl learning. Specifically, if pups

#### **TABLE 15.1**

## Infant Rats Show at Least Three Types of Learning in Early Life and Each Type of Learning Is Influenced by Maternal Presence, Albeit in Unique Ways

Type of Learning	Age Range	<b>Behavior Learned</b>	Brain Areas Used		
Attachment learning	PN1-9	Odor preference and social interaction with mother	LC, olfactory bulb, and anterior piriform cortex		
Maternal presence	Extends this learning to PN16				
Fear learning	≥PN10	Odor avoidance, freezing	Posterior piriform cortex and amygdala		
Maternal presence	Delays emergence of fear to PN16				
Infant malaise learning	Fetal to ~PN16–18	Odor avoidance	Olfactory bulb and piriform cortex		
Maternal presence	Fetal to ~PN16-18	Blocks learning			
Note: The unique brain areas used for infant learning are also illustrated.					

nurse during odor-LiCl conditioning, they develop a preference for the odor (Shionoya et al. 2006), while taste-LiCl avoidance learning is blocked (Martin and Alberts 1979; Gubernick and Alberts 1984; Melcer et al. 1985; Kehoe and Blass 1986).

## 15.4 IMPACT OF EARLY LIFE EVENTS ON ADULT ODOR MEMORY

Early life experiences have a profound effect on later life sensory, cognitive, and emotional processing. While a wide range of manipulations has been used to manipulate early life experiences (maternal deprivation, handling, maternal licking, odor-shock), there is remarkable convergence concerning mediating factors to induce enduring adult effects. Indeed, long-term effects have been documented in the hypothalamic–pituitary–adrenal (HPA) axis and the extended limbic system, including the LC, hippocampus, amygdala, and prefrontal cortex (Plotsky et al. 2005; Francis et al. 1999; Dent et al. 2001). While olfactory learning has not received much attention, adult cognitive function is well documented to be increased or decreased based on the type and age of infant manipulations (Fleming et al. 1999; Pryce et al. 2003; Denenberg and Bell 1960; Caldji et al. 1998, 2000; Coplan et al. 1996; Denenberg 1963, 1999; Kosten et al. 2005, 2006; Levine 1962, 1967, 2001; Lindholm 1962; Sevelinges et al. 2007, 2008; Seckl and Meaney 2004; Romeo et al. 2009).

We have been assessing the effects of infant odor learning on adult odor learning. However, as we assess the enduring effects of infant odor learning, it is important to remember that odor attachment learning differs from adult learning in that it involves social learning and attachment that occurs within the unique functioning of the infant brain. Specifically, the novel odor paired with either stroking or 0.5 mA shock takes on characteristics of maternal odor to support approach responses and complex motor responses such as nipple attachment. This odor produces a profound effect in infancy, which is retained into adulthood where the odor enhances male and female sexual behavior (Moore et al. 1996; Raineki et al. In progress; Coopersmith and Leon 1986; Fillion and Blass 1986; Sevelinges et al. 2007).

The attachment odor learned in infancy also modifies odor learning in adulthood (Sevelinges et al. 2007, 2008). Specifically, early life paired odor-shock conditioning attenuates adult fear conditioning and alters the functioning of olfactory sensory cortical areas, such as the piriform cortex and the basolateral amygdala. However, other changes were found even when the conditioned odor was not present. Specifically, using field potentials induced in the piriform cortex and the basolateral

amygdala in response to paired-pulse stimulation of the olfactory bulb revealed that in these sites, the level of inhibition was significantly reduced in adult rats, but only if they had received infant paired odor-shock conditioning and not unpaired. These results suggest that the contingency or predictability of early life experience are important variables for an enduring effect of early life experience on adult cognition. Thus, infant experiences enduring effects are due, at least in part, to learning effects that interact with neural changes induced through experience to alter the trajectory of brain development.

#### 15.5 CONCLUSION

This review of the neurobiology of emotional olfactory memory has focused on rat odor fear conditioning and highlights the fact that the olfactory modality might be particularly appropriate for understanding the interplay between limbic and cortical areas for the acquisition and storage of learned associations. Indeed, the existence of rapid direct anatomical connections between early sensory areas (i.e., the olfactory bulb and the piriform cortex) and the amygdala seems to favor the implication of olfactory cortical areas at early stages of learning acquisition. Together, these properties might endow the sense of smell with the capacity to automatically trigger emotional odor memories.

The developmental research reviewed here also illustrates the remarkable functional changes that occur in learning as an animal makes the transition from one developmental phase to the next. During early life, when infant rats are confined to the nest, they rapidly learn to prefer an odor when it is paired with myriad stimuli that appears dependent upon their ability to raise NE levels within the bulb rather than their hedonic value. This unique early life learning system may have evolved because of the critical survival value of odor learning: pups must learn the maternal odor to approach the mother and nipple attachment (Polan and Hofer 1999; Pedersen and Blass 1982; Leon 1992). Therefore, pups' survival is dependent on odor approach learning, suggesting that evolutionary pressure may have developed a neural circuit to ensure pups only learn to approach their caregiver regardless of the quality of care received (Bowlby 1965; Hofer and Sullivan 2001). The development of odor learning provides insight into at least a few basic principles concerning the development of the neurobiology of learning: (1) learning that appears similar throughout development can be supported by neural systems showing very robust developmental changes; (2) the functional emergence of the amygdala, or perhaps any brain structure, during ontogeny depends upon which function of the brain area is being assessed; and (3) the context of learning (maternal presence) dramatically modifies the neural circuitry pups use for odor learning, and hence the behaviors learned.

The olfactory system is an evolutionarily old sensory system with a simplistic neural circuitry, at least compared to other sensory systems, that has direct access to the emotional centers of the brain. This suggests that the sense of smell has a critical role in controlling emotional behavior that has been maintained through evolution. Environmental odors control emotions and behavioral responses that enable avoidance of danger but approach in social situations, both of which enhance survival of the individual and the species. Importantly, the olfactory system is remarkably flexible and eloquently designed for emotional learning and memory, which is reflected in odors' direct access to brain areas critical for emotional learning. Finally, the remarkable flexibility of the olfactory system to adapt to divergent learning demands at different developmental periods during the life span underscores the critical role of this sensory system in emotional and social attachment.

#### ACKNOWLEDGMENT

This work was funded by grants NICHD-HD33402, NSF IOB-0544406, NIDCD-CD009910, NIMH-MH086952, and Oklahoma Center for Science and Technology OCAST to RMS, and by grants ANR-05-PNRA-1.E7 AROMALIM to AMM.

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# 16 New Perspectives on Olfactory Processing and Human Smell

Gordon M. Shepherd

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Our understanding of the neural basis of olfactory processing is in a strong growth phase, with active research at all levels of the system. This research will not only provide insights into each stage of processing, but also a framework for understanding how the system functions as a whole.

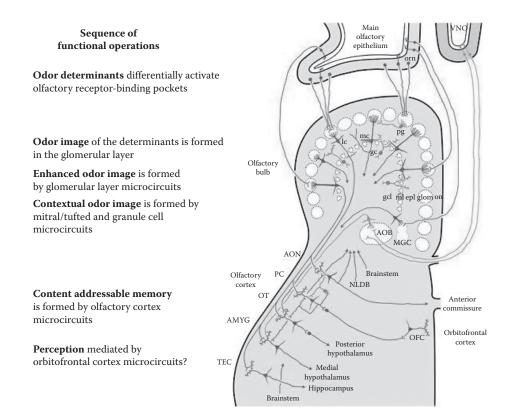
Most of this research has been carried out in animals. Many of the principles in the vertebrate also apply in the invertebrate (cf. Wilson 2008). In this chapter, I will briefly review the state of our knowledge of these steps, and then ask to what extent this work in animals gives us new insights into human olfaction.

## 16.1 THE BASIC STEPS OF OLFACTORY PROCESSING

Our current understanding of the neural basis of olfactory processing in the olfactory pathway is summarized in Figure 16.1.

## 16.2 THE DUAL OLFACTORY SYSTEM

Obviously, the first step is delivery of odorous molecules to the olfactory epithelium. Relatively little attention has been paid to this step, compared with the considerable amount of knowledge about how sensory stimuli reach their sensory receptors in other systems (i.e., the optics of the eye in vision, and the outer and middle ear in audition).



**FIGURE 16.1** (See color insert following page 206.) Summary of neural steps in processing of olfactory sensory stimuli as the basis for smell perception in the mammal. Abbreviations: ORN, olfactory receptor neuron; pg, periglomerular cell; mc, mitral cell; tc, tufted cell; gc, granule cell; on, olfactory nerve layer; glom, glomerular layer; epl, external plexiform layer; ml, mitral cell body layer; gcl, granule cell layer; AOB, accessory olfactory bulb; MGC, modified glomerular complex; AON, anterior olfactory nucleus; PC, piriform cortex; OT, olfactory tubercle; AMYG, amygdala; TEC, transitional entorhinal cortex; OFC, orbitofrontal cortex.

Traditionally, the focus has been almost entirely on stimulation by inhalation of vapors from the outside air, the so-called orthonasal route. Recently, attention has begun to be directed to the retronasal route, by exhalation while consuming food in the mouth. We will take this up later in dealing with human olfaction.

#### **16.3 LIGAND-RECEPTOR TRANSDUCTION**

The first neural step is the action of odor molecules on olfactory receptors in the cilia of the olfactory receptor cells. The receptors in mammals were shown by Buck and Axel (1991) to be a large subfamily of G-protein-coupled receptors (GPCRs). In analogy with how other GPCRs function, odor molecules are believed to interact within a binding pocket in the receptors (Singer 2000; Araneda et al. 2000), except that the odorant receptors (ORs) appear to have broader response spectra (molecular receptive ranges: MRRs). By these interactions, the information carried in the odor molecules is transferred into the neural domain.

The ORs provide attractive models for analysis of structure–activity relations (SARs) of a family of membrane receptors. Building on the early work, current studies using computational molecular modeling are providing evidence for the determinants of the odor molecules that interact with specific sites in the binding pockets (Schmuker et al. 2007; Khan et al. 2007). This is an essential step toward understanding how relationships between odor molecular structures are represented in an

efficient manner in the neural domain. These studies are beginning to predict, from odor molecule structure, the responses of olfactory cells and the relative pleasantness of the perception.

## 16.4 REPRESENTATION OF MULTIDIMENSIONAL ODOR SPACE IN THE TWO-DIMENSIONAL OLFACTORY GLOMERULAR SHEET

The subset of olfactory receptor cells expressing a given receptor projects its axons, in most cases in the mammal, to two glomeruli on the medial and lateral hemispheres of the olfactory bulb (Vassar et al. 1994; Ressler et al. 1994). The glomeruli are key structural modules in reflecting the MRRs of the receptors. The rat olfactory glomerular sheet consists of some 2000 glomeruli. A working hypothesis is that this sheet is generally a two-dimensional representation of the multidimensional odor space. The functional evidence that there would be a spatial representation of odor molecules began with Adrian (1953). The evidence for the spatial representation in the glomerular sheet began with the use of the activity label 2-deoxyglucose (2DG), which showed that odor stimuli gave rise to spatial patterns of activity distributed widely in the glomerular sheet (Sharp et al. 1975; Stewart et al. 1979). These patterns were different, but overlapping, with different odors, and increased from single glomeruli at threshold to large domains with increasing stimulus concentration. These basic features have been confirmed and extended by multiple types of activity markers—voltage sensitive dyes, immediate early gene expression, intrinsic imaging, Ca imaging, etc.—in the intervening years, in both vertebrates and invertebrates (reviewed in Xu et al. 2000).

Although the spatial activity patterns representing odor stimuli appear to be well established, the mechanisms underlying this representation are only beginning to be revealed. The role of temporal patterns in the responses is being vigorously investigated (Laurent 2002). Optical imaging of dorsal glomeruli shows that different individual glomeruli are activated by different odor stimuli, but how this relates to the larger patterns on the medial and lateral surfaces is not clear. More extensive optical imaging shows that there is a tendency for clustering of responses to similar odors (Mori et al. 2006), yet other studies indicate widespread representation of a given odor in the glomerular sheet (Soucy et al. 2009).

A continuing problem is the degree of reproducibility of a given pattern in a given animal, and the conservation of a given pattern across trials, individuals, and species. The fact that this appears to be only approximate has been the source of concern among some investigators, who propose that a spatial pattern must have precise spatial coordinates in order for space to be used to represent accurately the odor world. However, this misunderstands the nature of space in this system. The question is not whether the location of a given site is stable across trials, animals, or species, but whether the relations between sites are stable; in other words, the sites may be shifted while still preserving their relations. The distorted image in a carnival mirror is an example. The redundancy built into the overlapping receptor spectra further relaxes the constraints on spatial sites and relationships.

## 16.5 THE GLOMERULUS AND THE ENERGY BUDGET FOR NEURAL SIGNALING

Within the glomerular layer, the initial stages of synaptic processing take place, first within the glomeruli, then between them. Intraglomerular processing involves axodendritic inputs from the olfactory receptor cell axon terminals onto the dendritic tufts of mitral, tufted, and periglomerular (PG) cell dendrites, followed by obligatory dendrodendritic and dendroaxonic interactions between mitral/tufted cell dendritic tufts and PG cell tufts (Pinching and Powell 1970a, 1970b).

The study of this stage of processing is on the increase. Anatomical studies are revealing a wealth of complex synaptic interconnections (Kosaka and Kosaka 2005). Physiological studies are revealing the active properties, including calcium transients, of the dendritic tufts (Zhou et al. 2006). This is believed to contribute to signal-to-noise enhancement to enable the mitral/tufted cells to respond to weak threshold odor concentrations (Shepherd and Chen 2007).

The energy demands of activity in these interconnections have been of interest. The pioneering work of Attwell and Laughlin (2001) in the cerebral cortex provided the first energy budget for neural signaling. The olfactory glomerulus recommended itself as an attractive site for further study using this approach, because it is so clearly anatomically defined. A quantitative study of membrane pumps in all the neural, glial, and vascular elements of the glomerulus indicated that, with maximal activation of the input, about half the pump energy is consumed by membrane potential maintenance; the other half is divided between action potential traffic in the olfactory receptor axons and their postsynaptic dendritic responses, with only a small portion required by dendrodendritic synaptic interactions (Nawroth et al. 2007). Further studies have shown that energy metabolism occurs in the olfactory nerve layer despite a low density of mitochondria, a mismatch requiring further research (Lecoq et al. 2009).

As shown by Attwell and Laughlin (2001), the high energy costs of action potentials suggests sparse coding, involving relatively small populations of active cells firing at low frequencies. This is a useful working hypothesis in future investigations of olfactory glomeruli. One suggestion is that odor encoding may be facilitated at low impulse frequencies by the high degree of convergence onto a glomerulus.

#### 16.6 COMPLEX PROCESSING IN THE GLOMERULAR LAYER NETWORKS

The first steps in processing the spatial patterns of activity in the glomerular layer are carried out by interactions between glomerular responses. Interglomerular processing is mediated by glomerular layer cells connecting through their axons to cells associated with other glomeruli, which may be at varying distances.

The nature of these interactions is the subject of considerable interest. They appear to involve lateral inhibitory effects that may mediate contrast enhancement and initial extraction of molecular features strength (Aungst et al. 2003). External tufted cells appear to establish complex networks that set the background level of excitability independent of odor stimulus (Cleland et al. 2007). Plateau potentials in these cells appear to play critical roles in these networks (Zhou et al. 2006).

## 16.7 LATERAL INHIBITION SHAPES THE OUTPUT FROM THE OLFACTORY BULB

From the glomerular dendritic tufts, the synaptic potentials spread to the mitral/tufted cell bodies to elicit action potentials, which carry the output of the olfactory bulb to the olfactory cortex and backpropagate into the lateral dendrites. There, they activate dendrodendritic synapses onto granule cell spines, which, in turn, feed back inhibition onto the excited cell as well as onto others to which that granule cell is connected (Rall et al. 1966; Rall and Shepherd 1968). This brings about lateral inhibition, which is believed to be involved in processing the spatial patterns elicited in the glomerular layer. This is one of the closest correlations known between lateral inhibition and the synaptic microcircuit that generates the inhibition in the nervous system.

Study of the dendrodendritic microcircuit has grown to constitute a field of its own. In the original model, it was proposed that the lateral inhibition could be activated by either passive spread or active impulse propagation in the lateral dendrite. Recordings with patch electrodes and Ca imaging have shown that full impulse propagation may occur throughout the length of the dendrite (Xiong and Chen 2002). The synaptic microcircuit is mediated primarily by NMDA receptors on the granule cell spines receiving the glutamatergic input from the mitral cell dendrites (Schoppa et al. 1998).

## 16.8 LATERAL INHIBITION IS CARRIED OUT BY DISTRIBUTED CELL COLUMNS

The original model assumed that lateral inhibition would be imposed on less active neighbors, but it did not indicate how extensive the surround might be. This has been investigated by using

pseudorabies virus to track the connectivity of mitral and granule cells. Rather than a continuous network of labeled cells, the results showed a system of columnar cell arrangements of varying thickness, distributed widely throughout the olfactory bulb (Willhite et al. 2006). The narrow column of labeled granule, mitral, tufted, and PG cells appears to be centered over a single glomerulus, which can be considered a "glomerular unit."

This arrangement took on functional meaning from an independent study modeling lateral inhibition mediated by the mitral-granule interactions. This showed that strong lateral inhibition over long distances required active impulse propagation in the lateral dendrites, as had been shown by Xiong and Chen (2002). It also required activation of granule cells in narrow columns that inhibited the mitral cells at or near their cell bodies. The two studies thus converged on the same modification of the original model: backpropagating action potentials activate distant mitral-granule-mitral dendrodendritic inhibition (Migliore and Shepherd 2007).

By this means, strong inhibition can be delivered by activated mitral cells on other mitral cells largely independent of distance. It is postulated that this enables lateral inhibitory processing of the spatially extended activity maps from the olfactory glomeruli. This can be referred to as "distributed contrast enhancement," equivalent to what has been termed "nontopographical contrast enhancement" (Cleland et al. 2007; Arevian et al. 2008), and in contrast to the continuous center-surround receptive fields in the visual system.

## 16.9 PARALLEL SUBSYSTEMS PASSING THROUGH THE OLFACTORY BULB

We note several subsystems devoted to different types of olfactory stimuli, which are discussed at length in other chapters.

The accessory olfactory bulb (AOB) (see Figure 16.1) receives input from the vomeronasal organ and sends it on to the amygdala. In rodents, it processes mainly pheromonal signals. Recent studies indicate that it is also activated by major histocompatibility complex (MHC) peptides. In fact, the vomeronasal organ also responds to ordinary odor molecules. From a phylogenetic perspective, the AOB is not exclusively devoted to pheromonal detection. In other mammals, the AOB is used for other kinds of behavior, such as prey detection in snakes.

The septal organ is a special region whose cells have been shown to share properties with the cells in the main olfactory epithelium (see Chapter 9).

A modified glomerular complex (MGC) was discovered (Teicher et al. 1979) nestled against the medial side of the AOB. The MGC turned out to be related to a string of glomeruli around the AOB, called "necklace glomeruli." These were shown to receive input from olfactory receptor cells that use cyclic guanosine monophosphate as their second messenger for olfactory transduction. They also show high levels of expression of acetylcholine.

## 16.10 ADULT NEUROGENESIS REPLACES BOTH THE OLFACTORY BULB INPUT AND ITS INTERNEURONS

In addition to these features of signal processing, the olfactory pathway is of general interest because of the presence of neurogenesis in the adult. This was found first for the olfactory receptor cells, which arise from basal cells in the olfactory epithelium (Graziadei and Graziadei 1985). Continual generation of new neurons was then found in the brain ventricles, with migration of neuron precursors in the rostral migratory stream to become granule cells and PG cells in the olfactory bulb (Luskin 1993).

From this perspective, the olfactory bulb must be one of the most plastic regions of the nervous system, even more so than the dentate gyrus. The projection neurons—mitral and tufted cells—appear to be the anchors for populations of input fibers, glomerular layer interneurons, and granule cells undergoing constant turnover. The mechanisms of cell differentiation of the rostral migratory stream, maturation, and incorporation into the populations of interneurons, are subjects of intense investigation (Lledo et al. 2008).

What is the reason for this continual cell turnover? The receptor cells are the only nerve cells directly exposed to the environment. It is postulated that noxious and infectious elements inhaled in the air cause continual cell loss, acting thereby, directly or indirectly, as mitogens to stimulate cell turnover and replacement. For the olfactory bulb interneurons, the reason is less clear. Possibilities are: continual adjustment to the changing receptor cell input, to pheromonal odors involved in maternal recognition and bonding, and to the high risk of infection spreading from the nasal cavity.

#### 16.11 OLFACTORY CORTEX AS A CONTENT ADDRESSABLE MEMORY

The output from the mitral/tufted cells is projected to the olfactory cortex by the axons in the lateral olfactory tract, from which collaterals make axodendritic glutamatergic synapses on the distal dendrites of cortical pyramidal cells. Within the cortex, recurrent collaterals excite GABAergic interneurons that feed back inhibition onto pyramidal cells; they also directly excite themselves and other pyramidal cell dendrites to bring about recurrent feedback and lateral excitation. This constitutes the basic circuit of three-layer cortex (Shepherd 2004).

The convergent–divergent network of connections makes the cortex a content-addressable memory system (Neville and Haberly 2004). This is important, because it means that there is great redundancy in the representation of odor molecules at this stage, much as in higher association areas in other sensory systems. Cortical cells have been shown to adapt rapidly to continued stimulation with a given odor (Wilson and Stevenson 2003).

That the olfactory cortex mediates an astonishing range of functions is not full appreciated. First and foremost, it is the primary receiving cortex for common types of odor molecules. In addition, recent studies show that the main olfactory bulb also processes pheromonal inputs (Schaefer et al. 2002; Xu et al. 2005). Some of this output may be carried by tufted cells to the olfactory tubercle. To the extent that this information is also projected to the olfactory cortex, it means that the olfactory cortex is able to separate common and pheromonal inputs and distribute them differentially to the neocortex for perception and to limbic areas for reproductive and related behaviors. This must be particularly true in the human, which apparently lacks a functional vomeronasal pathway.

In addition, the olfactory cortex has been shown to be the site in the brain that monitors the consumption of essential amino acids in the diet. This involves sensitivity of the cortical cells to charging of transfer RNA with the appropriate amino acids; if lacking in the chow, a rodent ceases eating it within a half hour (Gietzen and Rogers 2006). There is evidence that the same mechanism may be acting in humans. Further studies of the olfactory cortex are summarized in Neville and Haberly (2004) and Wilson and Linster (2008).

## 16.12 ORBITOFRONTAL CORTEX (OFC): THE NEOCORTICAL PRIMARY OLFACTORY CORTEX

The olfactory cortical output is carried in the pyramidal cell axons. Some of this output goes through collaterals as centrifugal fibers back to the olfactory bulb; some to cells of the endopyriform nucleus; and some directly to the orbitofrontal cortex (OFC), which represents the primary sensory cortex at the neocortical level for the olfactory system. The endopyriform nucleus is believed to provide a pathway to the OFC through mediodorsal thalamus. The direct pathway means that olfaction is the only major sensory system that has direct input to the neocortex without passing through the thalamus. This combines with the fact that olfaction is the only major sensory system that has direct access to the OFC. The OFC is well known as belonging to the prefrontal cortex, the highest integrative level of the brain, responsible in large part for the higher cognitive capabilities of primates and especially humans.

Anatomical studies have revealed the complex circuits of which the OFC is a part. These are divided into sensory pathways that provide input from most of the major sensory systems, and motor pathways to the amygdala and other limbic structures (Ongur et al. 2003), making the OFC a critical node for multisensory integration. Physiological studies of the olfactory receiving cortex have shown the high degree of plasticity of cell responses in the OFC to odor stimuli (Alvarez and Eichenbaum 2002). Studies in primates have shown that single cells show reversal learning, the ability to change their response patterns in conditioning paradigms (Rolls 2005). Further studies using functional imaging in humans are described below. Thus far, there is little evidence for the intrinsic circuit organization within the OFC.

Study of the OFC is at an early stage compared with the lower levels of the olfactory pathway, and may be regarded in many ways as the last frontier for the neural basis of olfactory perception (see also below).

#### 16.13 RELEVANCE FOR HUMAN SMELL PERCEPTION

The studies reviewed above indicate that there is increasing evidence from research in laboratory animals for each step along the way from breathing in to perceiving a smell. What is the relevance for human smell perception?

Traditionally, it is believed that human smell perception is much reduced compared with other mammals, so that whatever abilities might be seen in animal research would have little significance for humans. However, that view is changing, from new evidence at many levels in the system. We note briefly some of these advances.

It was pointed out many years ago in human psychophysical studies that olfaction is not one but two systems (Rozin 1982). The most obvious is when we breathe in, called orthonasal olfaction. Less obvious is when we consume a food, and while it is in our mouths, sense its flavor. This requires breathing out, so that the vapors from the food pass through the nasopharynx to the nasal cavity, called retronasal olfaction.

Until recently, almost all animal research in olfaction was carried out on orthonasal olfaction. It is only in recent years, especially in human flavor research, that evidence has begun to be obtained for retronasal olfaction. These studies have made it clear that, for humans, in the sensing of flavor, retronasal smell has a large role to play in human behavior.

With regard to ligand-receptor interactions, we are still a long way from understanding the significance of the numbers of ORs and olfactory receptor neurons (ORNs). The fact that humans have only some 350 functional OR genes, compared to over 1000 in mouse or rat, has been taken to indicate the decline of olfaction in humans. However, dogs have only some 850, though having outstanding senses of smell, traditionally believed to be due to their large numbers of ORNs. The numbers of functional ORs are therefore only one variable in determining smell acuity, either in detection thresholds or odor discrimination ability.

At the level of the glomeruli, it is assumed that the rodent model applies to the representation of odor molecules in the glomerular sheet of the human. However, there is recent evidence that, rather than fewer glomeruli, to go with the lower number of ORs, the human olfactory bulb contains several thousand small glomeruli (Maresh et al. 2008). Thus, the significance of the numbers of glomeruli for olfactory perception also requires further study.

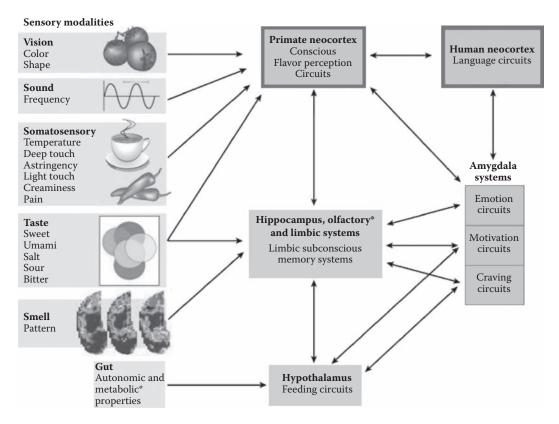
It is assumed that in the primate, the intrinsic circuits for processing within and between glomeruli, and at the level of mitral and granule cell interactions, are similar to those revealed in the rodent.

The olfactory bulb in monkeys is attached to a prolonged stalk containing the lateral olfactory tract. It raises the question of whether there is movement of cells belonging to the rostral migratory stream through the stalk to become granule and PG cells in the olfactory bulb. It has also been questioned whether a central ventricle exists for this movement in the primate olfactory bulb.

The model for the cellular and circuit organization of the olfactory cortex in rodents is assumed to apply to the primate, including human. The olfactory cortex is the first structure in the olfactory pathway that can be visualized in functional imaging; the olfactory bulb is too close to the underlying bone. Thus far, only general levels of BOLD signals can be seen in the olfactory cortex. There is no evidence in the rodent or human for differential spatial patterns of activation by odor stimuli within the olfactory cortex.

At the neocortical level, primates, and especially humans, come to the fore as experimental subjects for olfaction, for several reasons. First, the medial and lateral OFC olfactory areas are at their largest anatomically, compared with the small slivers of cortical tissue in rodents. Second, the complex sensory and motor systems of which the OFC is a part are at their highest development. Third, single-cell recordings in awake behaving animals are most effective, giving the best insights into the higher order learning capabilities at this level. Finally, human functional imaging studies are highly effective in exploring the complex relations between olfaction and other sensory systems, as well as emotional centers and higher cognitive functions.

Some of these relations are depicted in Figure 16.2. Although retronasal smell is the dominant sense in the perception of flavor, that perception is a multimodal one. Retronasal smell is always accompanied by stimulation of the taste buds on the tongue and oropharynx. Trigeminal fibers are activated, mediating the many submodalities of the somatosensory system, telling us whether a food is creamy, crunchy, hot or cold, painful, astringent, etc. Retronasal smell is also an active sense, always requiring extensive motor engagement, of the tongue, jaw, swallowing, exhalation, and all other types of motor control related to feeding behavior. Even before we begin eating, during the so-called "cephalic phase" of feeding, the visual qualities of a food affect our eventual perception of flavor. This has been shown most dramatically in a wine tasting of red and white wines, in which some of the whites had been colored red; they were judged to be red, even by professional wine tasters! Finally, even the sound of the food being consumed ("snap, crackle, and pop") affects the



**FIGURE 16.2** (See color insert following page 206.) Summary of the relation of the spatial representations of odor molecules to the multiple sensory modalities and central brain regions and pathways in the human that constitute the brain flavor system that is unique to the human. \*Sensors for essential amino acids. (From Shepherd, G. M., *Nature*, 444, 316–21, 2006. With permission.)

flavor sensation and palatability. Given the anatomical evidence cited above, presumably the OFC is a major center for combining these sensory sensations into the multimodal perception of flavor.

In addition, flavor has strong emotional qualities related to the pleasure of what is being sensed. The combined evidence from rodent, monkey, and human data indicate that these emotional qualities range from the relative pleasure of the flavor, to the level of motivation toward consuming foods with those flavors, to a craving for them. Cravings may range from those known to chocolate lovers, to addictions, and to substances of abuse such as tobacco and alcohol. The distributions of activated brain areas in subjects exposed to these substances have been called "images of desire" (Pelchat et al. 2004).

Figure 16.2 makes it clear that the olfactory system in humans is greatly elaborated by the enlarged human brain and, within it, the close and complex interactions with the powerful mechanisms for emotion and cognition. It is this enormous elaboration of brain power that offsets the decline in numbers and sensitivity of the olfactory receptors and receptor cells to amplify the sense of flavor. A similar increase in brain power applies to the human auditory system. Humans have only some 20,000 hair cells on a side, and a frequency range up to only 20 kHz, but they nonetheless have elaborated speech and language, whereas the moustache bat, with acute sensitivity and a frequency range up to 100,000 kHz, has elaborated a largely subcortical ability for sonar prey detection. The processing mechanisms of the human brain flavor system can therefore be viewed as one of our highest faculties in the struggle for survival during human evolution. From this and many other perspectives, smell perception and the human brain flavor system are the last frontiers of olfactory research.

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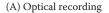
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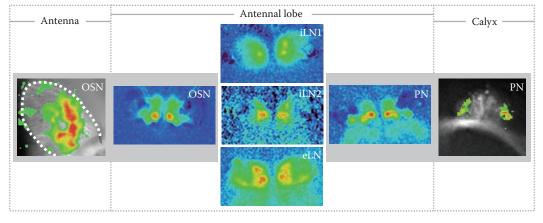
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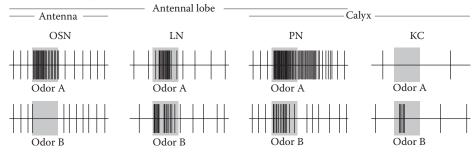
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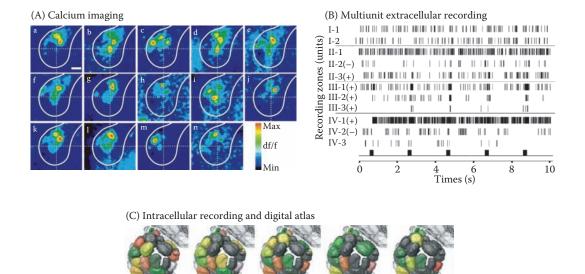




(B) Electrophysiology



**FIGURE 2.2** Examples for odor-evoked activity in the fruit fly. (A) (*left*) Responses to the odor isoamyl acetate in OSNs on the antenna (their dendritic compartments) and in the antennal lobe (their axonal compartments) are shown left. The calcium sensor was expressed under Or83b control. (*Center*) Responses to the same odor in two inhibitory (iLN1, iLN2) and one excitatory (eLN) local neuron line within the AL. (*Right*) Responses to the same odor in the mushroom bodies (their axonal compartment). For the antennal lobe (their dendritic compartments), and in the mushroom bodies (their axonal compartment). For the antenna and the mushroom body, nonactive areas were removed to visualize the underlying morphology. (B) Schematic view of characteristic activity patterns as spike trains, following the same sequence as in A, i.e., OSN, LN, PN and the mushroom body intrinsic KCs. Note the sparse firing properties of KCs as compared to PNs.



**FIGURE 3.8** Spatiotemporal organization of odor-evoked activity in the moth AL. (A) Olfaction activation pattern in the AL of *Spodoptera littoralis* revealed by calcium imaging. (B) Ensemble olfactory response in the AL of *Manduca sexta* revealed by tetrode recording. (C) Reconstructed geometry of odor-evoked firing activity in the AL of *Bombyx mori*. Anterior and posterior views of reconstructed population activities in response to cis-3-hexen-1-ol. The color of each glomerulus represents the average firing rate from baseline of the innervating PNs. ([A] From Carlsson, M. A., Galizia, C. G., and Hansson, B. S. *Chem. Senses*, 27, 231–44, 2002. With permission. [B] From Lei, H., Christensen, T. A., and Hildebrand, J. G. *J. Neurosci.*, 24, 11108–19, 2004. With permission. [C] From Namiki, S., and Kanzaki, R., *Front. Neural Circuits*, 2, 1, 2008. With permission.)

250-300 ms

450-500 ms

650-700 ms 850-900 ms

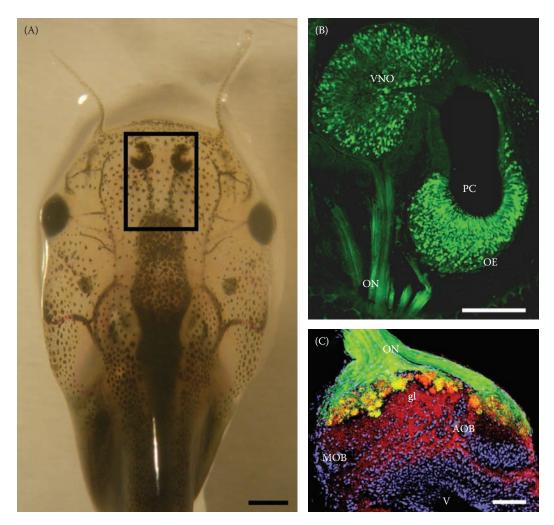
0

1050-1100 ms

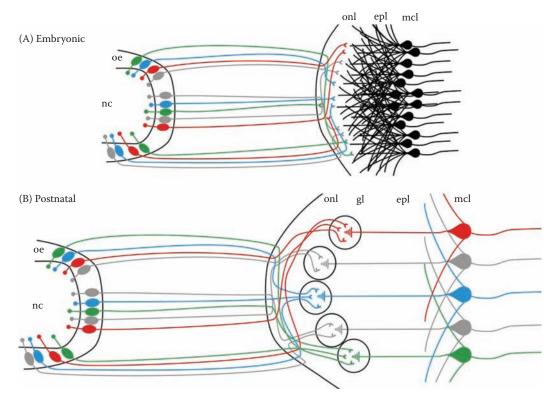
100

50

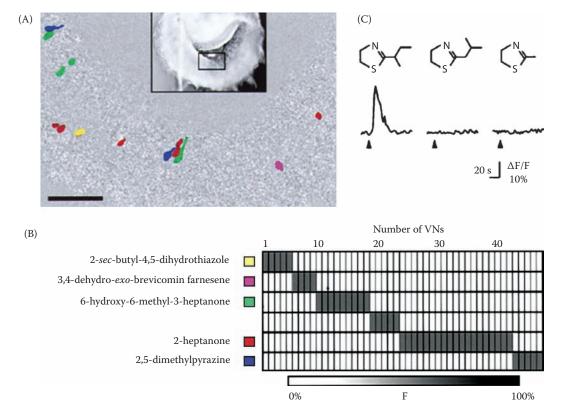
Firing rate (Hz)



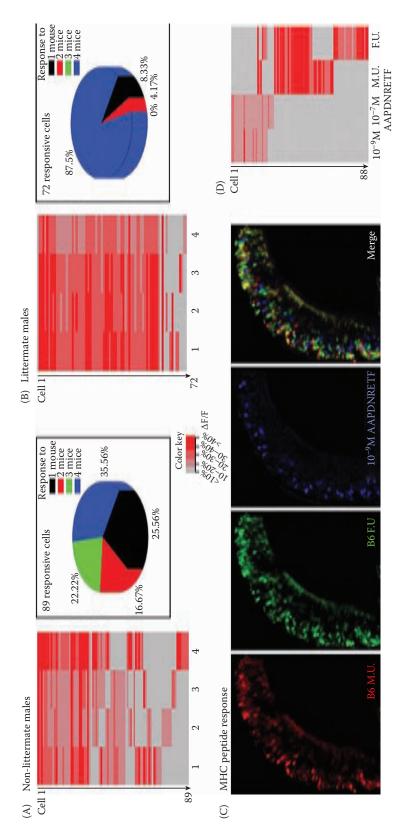
**FIGURE 4.1** The main and accessory olfactory system of larval *Xenopus laevis*. (A): Larval *Xenopus laevis* (stage 51) The black rectangle outlines the first two stages of the olfactory system (scale bar 2 mm). (B): Horizontal overview over the olfactory epithelium and the vomeronasal organ (VNO, vomeronasal organ; PC, principal cavity; OE, olfactory epithelium; ON, olfactory nerve). The neurons were backfilled through the olfactory nerve using biocytin/avidin staining (green fluorescence) (scale bar 200  $\mu$ m). (C): Horizontal overview over the olfactory nerve; MOB, main olfactory bulb; AOB, accessory olfactory bulb; GL, glomerular layer; V, ventricle). Biocytin injection into the olfactory nerve (green fluorescence), synaptophysin immunostaining (red fluorescence), and DAPI nucleic acid staining (blue fluorescence) (Scale bar 100  $\mu$ m). ([C] modified from Nezlin et al. *J. Comp. Neurol.*, 464, 257–68, 2003.)



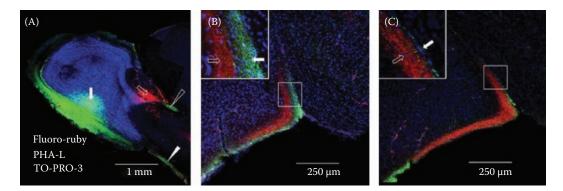
**FIGURE 5.3** Schematic diagram comparing embryonic (A) and postnatal (B) olfactory connectivity. During the postnatal period, OSNs express a single OR from a single allele (monoallelic expression, represented by different colors), and extend a single unbranched axon back to the olfactory bulb in large intermingled axon fascicles. OSN axons remain restricted to the ONL, directly apposed to a dense meshwork of dendrites of the developing projection neurons, the mitral and tufted cells. Mitral/tufted cell dendrites are very immature at this stage, with each neuron having multiple broadly spread apical dendrites. In the postnatal period, OSN axons have sorted out into "like-types" and targeted specific glomeruli. Mitral and tufted cells have undergone extensive remodeling to achieve their mature morphology of a single apical dendrite, which ramifies as an apical tuft within a glomerulus and numerous lateral dendrites that extend within the EPL. Abbreviations: nc, nasal cavity; oe, olfactory epithelium; mcl, mitral cell layer; epl, external plexiform layer; gl, glomerular layer; onl, olfactory nerve layer.



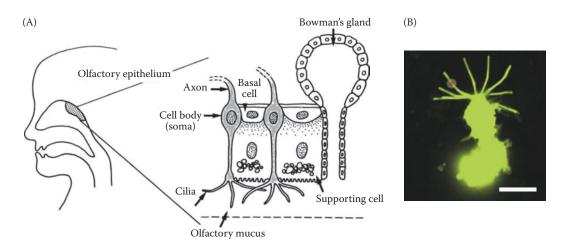
**FIGURE 6.1** Stimulus selectivity of mouse vomeronasal class 1 (V1R)-expressing vomeronasal sensory neurons (VSNs) recorded by Ca<sup>2+</sup> imaging from slices of the vomeronasal epithelium. (A) VSNs that responded to volatile pheromones were located in the apical region of the vomeronasal epithelium (area of image is shown by the box in the inset). Different response specificities are shown in different colors. (B) VSNs responded highly selectively to a single urinary volatile with known pheromonal activity. (C) A VSN that responded to 2-*sec*-butyl-4,5-dihydrothiazole failed to respond to stimuli with similar chemical features. (Reprinted by permission from Macmillan Publishers Ltd: [Nature] Leinders-Zufall, T. et al. *Nature*, 405, 792–96, copyright, 2000.)



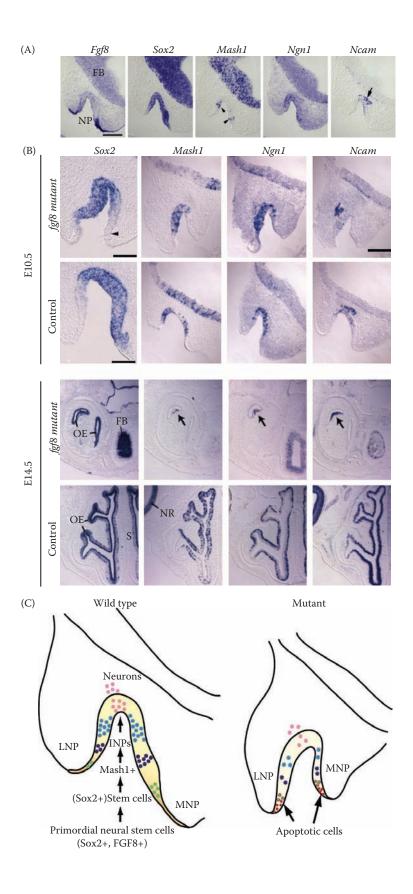
(MHC) peptides. Responses recorded by Ca<sup>2+</sup> imaging from slices of vomeronasal epithelium in response to urine from individual urine donors. Pie charts show percentage of VSNs that responded to different numbers of individuals. Differences in the patterns of response to individual males of the same strain suggest that the vomeronasal system is potentially sensitive to individual differences unrelated to genotype. (A) Responses to non-littermate males of the C57/BL6 inbred strain. (B) Responses to littermate males of the C57/BL6 inbred strain. (C) Images of VSNs that responded to urine from C57/BL6 males (B6 M.U.), C57/BL6 females (B6 F.U.), and the C57/BL6 MHC peptide FIGURE 6.2 Differences in the patterns of responses of vomeronasal sensory neurons (VSNs) to urine from different individuals and major histocompatibility complex AAPDNRETF. (D) The populations of VSNs responding to urine from C57/BL6 males and females showed little overlap with the population of VSNs responding to the C57/ BL6 MHC peptide AAPDNRETF. This suggests that this peptide is unlikely to be an endogenous individuality signal of mouse urine. (From He, J. et al. Science, 320, 535–38, 2008. Reprinted with permission of AAAS.)



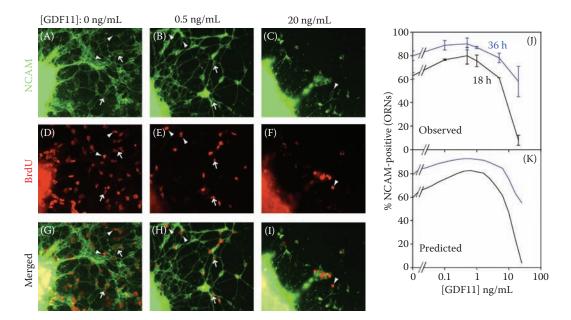
**FIGURE 6.3** Convergence of input from the ventral main olfactory bulb (MOB) and the accessory olfactory bulb (AOB) onto the medial amygdala (MeA) of the female mouse. (A) Location of injections of anterograde tracer into the ventral MOB in green, shown by filled arrow, and AOB in red, shown by open arrow. Convergence of projections from MOB (green, filled arrow) and AOB (red, open arrow) onto neighboring laminae in the anterior region of the MeA (B) and posterodorsal subdivision of the MeA (C). (Reprinted from Kang, N., Baum, M.J., and Cherry, J.A., *Eur. J. Neurosci.*, 29, 624–34, 2009. With permission from Wiley-Blackwell.)



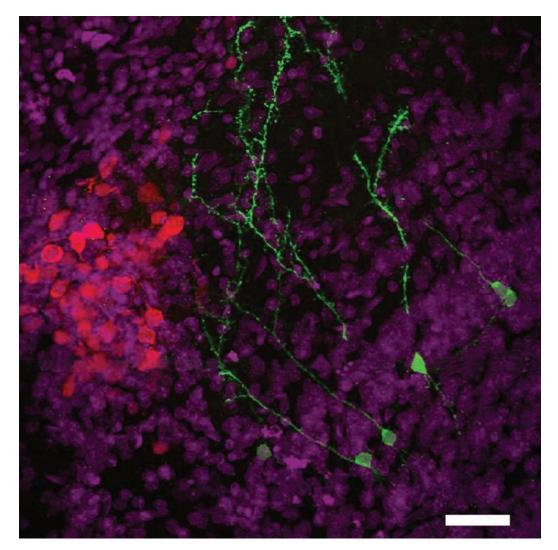
**FIGURE 8.1** Olfactory epithelium and olfactory receptor cell. (A) Schematic diagram of the localization of the human olfactory epithelium in the upper part of the nasal cavity (left panel). Schematic diagram showing the histological organization of the olfactory epithelium. Olfactory receptor cells (ORCs) are in gray. (B) Laser scanning microscope image of Lucifer yellow fluorescence loaded into an ORC isolated from a newt. In the picture, the ciliary image is thicker than the original diameter, because of the light deflection. The colored circle on a cilium indicates the area illuminated by a laser beam to photolyse caged cAMP previously loaded into the cell. Scale bar is 10 µm. (Modified from Takeuchi H and Kurahashi T, *J. Neurosci.*, 28, 766–75, 2008. With permission.)



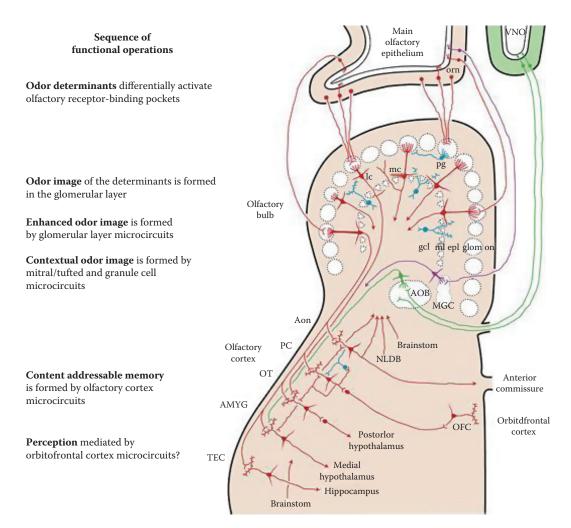
**FIGURE 10.3** (Opposite) Absence of Fgf8 leads to apoptosis of primordial Sox2-expressing OE neural stem cells and cessation of OE neurogenesis and nasal cavity morphogenesis. (A) Expression of Fgf8 and neuronal lineage markers in E10.5 olfactory pit (in situ hybridization, ISH). Note overlap between Fgf8 and Sox2 expression domains. Arrowheads: Mash1-expressing cells; arrow: Ncam-expressing neurons. FB, (presumptive) forebrain; NP, nasal pit. Scale bar: 200 µm. (B) Cessation of neurogenesis in Fgf8 mutants (conditional allele of Fgf8 deleted using BF1-Cre) (Hebert and McConnell 2000). Arrowhead marks reduced Sox2 expression in the OE lining the nasal pit at E10.5; arrows indicate apparent OE remnant in E14.5 mutant animals. FB, forebrain; NP, nasal pit; NR, neural retina; OE, olfactory epithelium; S, nasal septum. Scale bars: 200 µm. (C) Schematic of FGF8's role in OE neurogenesis. The sketch shows the relative positions of different neuronal cell types within the OE during primary olfactory neurogenesis at E10.5 in wildtype and Fgf8 mutants. Fgf8expression domain, orange; Sox2 expression domain (definitive neuroepithelium), yellow; Sox2+ stem cells, green; Mash1+ early progenitors, dark blue; Ngn1+ INPs, light blue; Ncam+ ORNs, pink. Cells in the Fgf8expressing domain that undergo apoptosis when  $F_{gf8}$  is inactivated are shown in red, and apoptotic primordial neural stem cells (Sox2+, Fgf8+) are in green with red jagged border. Vestigial populations of other neuronal cell types are shown in their corresponding colors, but with jagged borders. ([C] Adapted from Kawauchi, S. et al. Development, 132, 5211-23, 2005.)



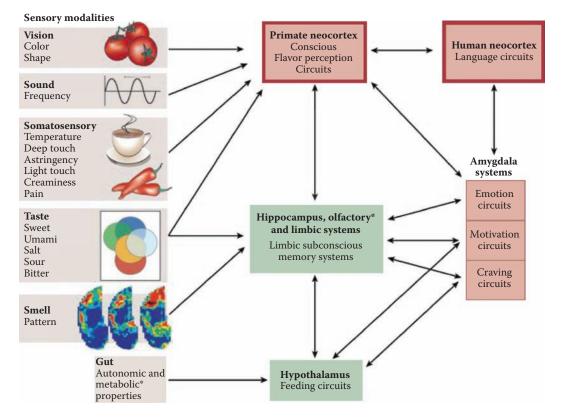
**FIGURE 10.5** GDF11 regulates ratio of INP proliferative vs. differentiative divisions. (A–J) At low doses of GDF11, the proportion of INP progeny that differentiate into ORNs increases. At high doses, the effect reverses, with the NCAM<sup>+</sup> fraction falling to near zero at 18 h, but recovering at 36 h. The reversal is consistent with a slowing of the cell cycle such that 18 h is insufficient for the production of NCAM-expressing, terminally differentiated ORNs (but 36 h is). This interpretation is consistent with previous data demonstrating that high doses of GDF11 reversibly arrest the INP cell cycle (Wu et al. 2003). (K) Simulation of the experiment in (J) by a model in which GDF11 affects both ratio of proliferative vs. differentiative divisions and division rate. ([K] Reprinted from Lander, A.D. et al. *PLoS Biol.*, 7, e15, 2009.)



**FIGURE 11.1** A newborn eGFP+ PG cell (green) around a glomerulus at P109, 3 weeks after virus injection. Calretinin-labeled (red) and tyrosine hydroxylase-labeled (blue) PG cells outline the glomerulus. Scale bar: 20 µm. (From Belluzzi, O. et al. *J. Neurosci.* 23, 10411–18, 2003. With permission.)



**FIGURE 16.1** Summary of neural steps in processing of olfactory sensory stimuli as the basis for smell perception in the mammal. Abbreviations: ORN, olfactory receptor neuron; pg, periglomerular cell; mc, mitral cell; tc, tufted cell; gc, granule cell; on, olfactory nerve layer; glom, glomerular layer; epl, external plexiform layer; ml, mitral cell body layer; gcl, granule cell layer; AOB, accessory olfactory bulb; MGC, modified glomerular complex; AON, anterior olfactory nucleus; PC, piriform cortex; OT, olfactory tubercle; AMYG, amygdala; TEC, transitional entorhinal cortex; OFC, orbitofrontal cortex.



**FIGURE 16.2** Summary of the relation of the spatial representations of odor molecules to the multiple sensory modalities and central brain regions and pathways in the human that constitute the brain flavor system that is unique to the human. \*Sensors for essential amino acids. (From Shepherd, G. M., *Nature*, 444, 316–21, 2006. With permission.)

The common belief is that human smell perception is much reduced compare to other mammals, so that whatever abilities are uncovered and investigated in animal research would have little significance for humans. However, new evidence from a variety of sources indicates this traditional view is likely overly simplistic.

**The Neurobiology of Olfaction** provides a thorough analysis of the state-of-the-science in olfactory knowledge and research, reflecting the growing interest in the field. Authors from some of the most respected laboratories in the world explore various aspects of olfaction, including genetics, behavior, olfactory systems, odorant receptors, odor coding, and cortical activity.

Until recently, almost all animal research in olfaction was carried out on orthonasal olfaction (inhalation). It is only in recent years, especially in human flavor research, that evidence has begun to be obtained regarding the importance of retronasal olfaction (exhalation). These studies are beginning to demonstrate that retronasal smell plays a large role in human behavior.

Highlighting common principles among various species—including humans, insects, *Xenopus laevis* (African frog), and *Caenorhabditis elegans* (nematodes)—this highly interdisciplinary book contains chapters about the most recent discoveries in odor coding from the olfactory epithelium to cortical centers. It also covers neurogenesis in the olfactory epithelium and olfactory bulb. Each subject-specific chapter is written by a top researcher in the field and provides an extensive list of reviews and original articles for students and scientists interested in further readings.



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