Neuron, Vol. 44, 31–48, September 30, 2004, Copyright ©2004 by Cell Press

Olfactory Learning

Review

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The olfactory nervous systems of insects and mammals exhibit many similarities, suggesting that the mechanisms for olfactory learning may be shared. Neural correlates of olfactory memory are distributed among many neurons within the olfactory nervous system. Perceptual olfactory learning may be mediated by alterations in the odorant receptive fields of second and/or third order olfactory neurons, and by increases in the coherency of activity among ensembles of second order neurons. Operant olfactory conditioning is associated with an increase in the coherent population activity of these neurons. Olfactory classical conditioning increases the odor responsiveness and synaptic activity of second and perhaps third order neurons. Operant and classical conditioning both produce an increased responsiveness to conditioned odors in neurons of the basolateral amygdala. Molecular genetic studies of olfactory learning in *Drosophila* have revealed numerous molecules that function within the third order olfactory neurons for normal olfactory learning.

Introduction

There are several reasons why the study of olfactory memory offers a particularly advantageous avenue for learning about the neurobiology of memories in general. The first is that there exists a very striking homology in the design and function of the olfactory nervous system between different classes of organisms, including insects and mammals. This offers reassurance that the principles established by studying one class of organisms will easily extend to others. Such design homology is much more difficult to discern for other sensory systems, such as the visual or somatosensory systems, when comparing representatives of the two classes or organisms. A second advantage for studying olfactory memories is that many experimental animals exhibit a very keen sense of smell and the ability to form olfactory memories. Rodents are particularly tuned to using olfaction for guiding their behavior. And, surprisingly, insects like *Drosophila*, which one would imagine to have keen visual memories due to their compound eyes that monitor the vast majority of their surrounding three-dimensional visual space, are particularly adept at olfactory tasks and olfactory learning. Any *Drosophila* researcher can attest to the fact that it takes only seconds for escapees from the fly room down the hallway to find a newly unwrapped sandwich on a researcher's office desk. In addition, many believe that olfactory memories are special in their own right (Chu and Downes, 2000; Savic et al., 2000). Odors can immediately alter affective states and arousal level, produce extremely vivid recall of associated emotional experiences, and persist for decades. This potency of odors can be a nice quality, by promoting pleasant olfactory memories, for instance, but it can also be a bad thing. Some olfactory memories are the unwanted switch that opens the door to mental illness such as drug addiction relapse and posttraumatic stress disorder. In posttraumatic stress disorder, highly charged, negative emotional events associated with an odor are reborn as vivid flashbacks so profound that they can be incapacitating (Vermetten and Bremner, 2003).

To understand how memories about odors are formed and stored in that part of the nervous system that is specialized for processing odor cues requires four basic steps. First, we must know about the principle cell types in the system, understand their connections, and have knowledge about how these neurons communicate with one another. Second, we must know how odors are encoded within the olfactory nervous system, that is, how different odors are represented in the system in terms of neural activity, biochemical changes, and any other alteration that serves the process of sensory encoding. We must also know how these olfactory representations are processed or transformed in their nature over time, and as they are routed between different olfactory neurons. Third, we must elucidate the changes that occur in the representations of odors due to learning. Each change, in principle, will reveal clues about the alterations in the olfactory nervous system that have occurred due to learning to provide for a different representation of the odor. Finally, we must dissect the system at the molecular and cellular level, to reveal the molecular building blocks of the olfactory nervous system and its principal working parts used for learning and memorizing odors.

This review is organized around these four steps in summarizing and evaluating our knowledge of olfactory learning. Several types of olfactory learning, however, are not discussed. These, in general, are those specific olfactory memories that form during critical periods of development or during life events, including the following: (1) the Bruce effect, in which the memory of pheromonal cues from a male mate formed during a critical period after rodent mating blocks the pregnancy termination effects produced by the pheromonal cues from a second male; (2) the olfactory memory formed by a ewe of her lamb from odor cues in the lamb's wool and skin after parturition, providing for sib recognition; and (3) the olfactory memory formed by neonatal rabbits and rats of maternal pheromones that aid in locating the mother's nipples for suckling. These critical period olfactory memories are discussed in several excellent reviews (Wilson and Sullivan, 1994; Brennan and Keverne, 1997).

Rather, I focus here on the everyday olfactory memories that are not dependent on critical periods and pheromonal cues, although the underlying mechanisms are

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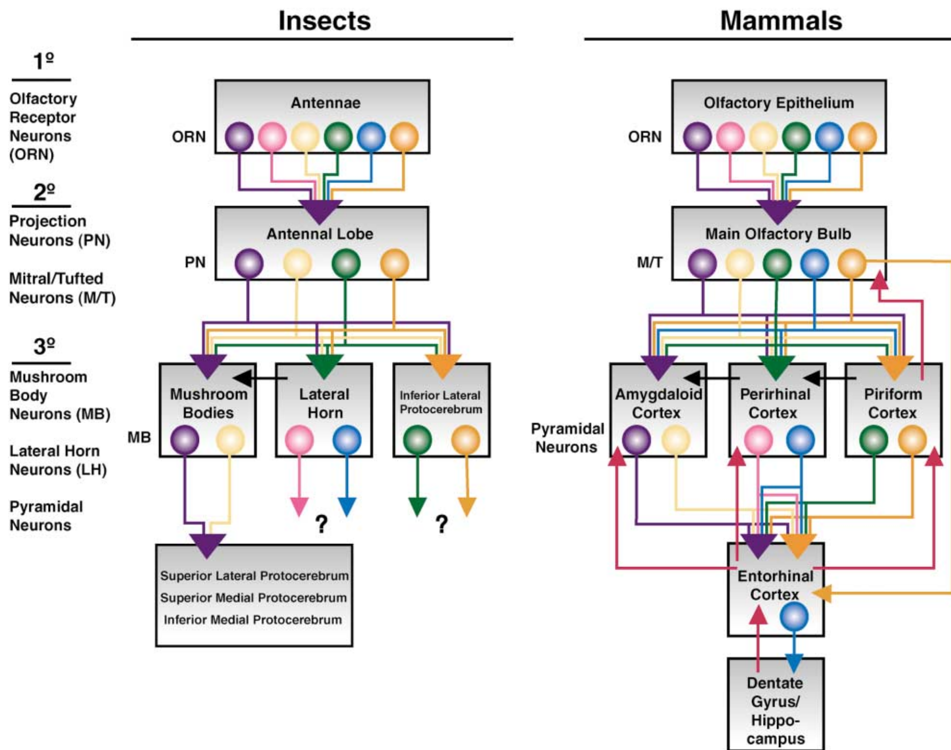

Figure 1. Hierarchy of the Olfactory Nervous System in Insects and Mammals

likely to be shared. These memories form from odor cues that are sensed and processed by the main olfactory epithelium and main olfactory bulb in mammals. I also focus on insects and mammals, because of obvious parallels and therefore the ability to draw comparisons. In this task, *Drosophila* and the rat/mouse are the subject of much of the discussion as "representatives" of the two animal classes, overlooking some species-specific differences and overgeneralizing about others in order to synthesize a broad perspective of olfactory learning.

The Olfactory Nervous System in Insects and Mammals

The anatomical organization of the insect olfactory nervous system shares many fundamental similarities with that of mammals (Brennan and Keverne, 1997; Hildebrand and Shepherd, 1997; Haberly, 1998; Laissue et al., 1999; Lessing and Carlson, 1999; Vosshall et al., 2000; Laurent et al., 2001; Mombaerts, 2001; Roman and Davis, 2001), suggesting that the mechanisms for olfactory perception, discrimination, and learning are shared (Figures 1–3). The neurons representing the interface between the environment and the nervous system are the 1° olfactory receptor neurons (ORNs), which reside in the antennae and maxillary palps of insects and in the olfactory epithelium of mammals. In *Drosophila*, about 1300 ORNs are distributed between the antenna and maxillary palp on each side of the head and project axons to the antennal lobe, where they terminate in ~43 morphologically discrete and synapse-dense processing modules known as glomeruli (Figure 2; Gao and Chess, 1999; Laissue et al., 1999; Vosshall et al., 2000; Scott et al., 2001). The projection patterns of the ORNs are stereotyped between animals; ORNs that express the same olfactory receptor gene, although distributed across the surface of the antenna and maxillary palps, project their axons to the same glomerular target in the antennal lobe (Gao et al., 2000; Vosshall et al., 2000; Scott et al., 2001). There, they are thought to form excitatory synapses with at least two classes of neurons, the local interneurons (LNs) and the projection neurons (PNs). The LNs are axonless, are thought primarily to be GABAergic inhibitory neurons, and have broad, multiglomerular ramifications within the antennal lobe (Leitch and Laurent, 1996; Sun et al., 1997; Laissue et al., 1999). A unique feature of the circuitry within the insect antennal lobe is the existence of reciprocal dendrodendritic connections between the PNs and the LNs (Sun et al., 1997; Didier et al., 2001; Ng et al., 2002). The presence of these unique junctions with both transmissive and receptive synapses provides anatomical evidence that each glomerulus makes computations that may underlie odor perception, discrimination, and learning, rather than being a simple transit station for the throughput of olfactory information. Individual PNs generally extend dendrites into a single antennal lobe glomerulus (Jefferis et al., 2001; Marin et al., 2002; Wong et al., 2002) and then convey the processed olfactory information to the 3° olfactory neurons (Figures 1 and 2).

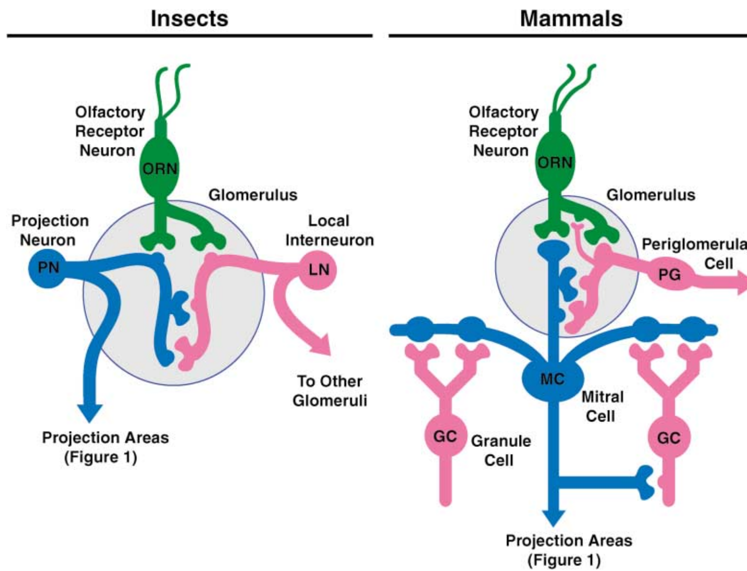

Figure 2. Synaptic Connections in the Glomeruli of the Antennal Lobe and Olfactory Bulb

Adapted from Laurent (2002), with permission.

The mammalian olfactory bulb has a strikingly similar organization, but more detail is known (Hildebrand and Shepherd, 1997; Mori et al., 1999). In the mouse, a few million ORNs that express one of ~1000 olfactory receptors are located in the olfactory epithelium and project to a few of the ~1800 possible olfactory bulb glomeruli, the specific glomerular target being dependent on which olfactory receptor is expressed in each neuron (Mombaerts, 2001). They synapse with interneurons and 2° olfactory neurons within the glomeruli. The 2° neurons in mammals and other vertebrates are the mitral/tufted (M/T) cells, and like the PNs of insects, the M/T cells form reciprocal synapses with GABAergic interneurons, including the periglomerular cells (PGs) and the granule cells (GC). The PGs form synapses with M/T neurons close to the terminals of the ORNs; the GCs form synapses on the lateral dendrites of the M/T neurons (Figure 2). Some periglomerular neurons are also dopaminergic and regulate ORN activity presynaptically through the release of this neuromodulator (Hsia et al., 1999; Ennis et al., 2001). The M/T neurons in mammals, like their PN counterpart in most insects, extend their apical dendritic fields into a single glomerulus and therefore receive direct olfactory information from their apical dendrites only from those ORNs that project to that same glomerulus. These neurons, however, have extensive lateral dendrites that project tangentially for long distances (Figure 2; Shepherd and Greer, 1998; Mori et al., 1999) and form the substrate for dual excitatory-inhibitory interactions with GCs and potentially mediate dendrodendritic excitatory interactions between pairs of mitral cells (Aroniadou-Anderjaska et al., 1999). Thus, the stimulation of a mitral cell in one glomerulus results in feedback inhibition on that cell from a stimulated GC, as well as inhibition or excitation (Aroniadou-Anderjaska et al., 1999) of mitral neurons in lateral glomeruli. Some PGs also provide inhibitory lateral interactions to the dendrites of neighboring M/T neurons (Mori et al., 1999), and short axon cells (data not shown) may provide for the inhibition of glomeruli distal to an excited M/T neuron (Aungst et al., 2003).

The piriform cortex is a three-layered cortical structure with primary inputs from the M/T neurons of the olfactory bulb (Figure 3A). These inputs are segregated into the lateral olfactory tract (LOT; dark blue), which runs along the surface of the piriform cortex and makes synapses with the apical dendrites of three types of pyramidal neurons (Figure 3). The M/T neurons that project from individual glomeruli in the olfactory bulb to the anterior piriform cortex have small and clustered but overlapping fields of termination within the cortex (Figure 3B; Zou et al., 2001). The same organization of terminal fields is found in the posterior piriform cortex, although the terminal fields are larger in area. These mapping data are consistent with the possibility that any individual odor will activate the same set of M/T neurons in the olfactory bulb in different animals and that these will then stimulate conserved clusters of piriform cortex neurons. They also open the possibility for piriform cortex neurons to integrate the information representing different odors that is conveyed by the overlapping terminal fields of M/T neurons (Wilson, 2001a). The overlapping mosaic pattern of terminal fields of neurons that project from different glomeruli is also reminiscent of the very distinctive and overlapping terminal field maps established by *Drosophila* PNs in the lateral horn (Marin et al., 2002; Wong et al., 2002; Tanaka et al., 2004). This similarity may suggest a homology between the piriform cortex of mammals and the lateral horn of insects. Such stereotyped maps of PN terminal fields also exist in the mushroom bodies, but they are more ambiguous (Marin et al., 2002; Wong et al., 2002; Tanaka et al., 2004).

A major difference between the primary olfactory system in mammals compared to other sensory systems is that there is no thalamic relay between the peripheral receptors and the primary olfactory cortex. This means that any information processing that occurs for other types of sensory information in the thalamus that may refine cortical receptor fields is either missing or compensated for by other mechanisms within the olfactory nervous system. However, like other sensory systems

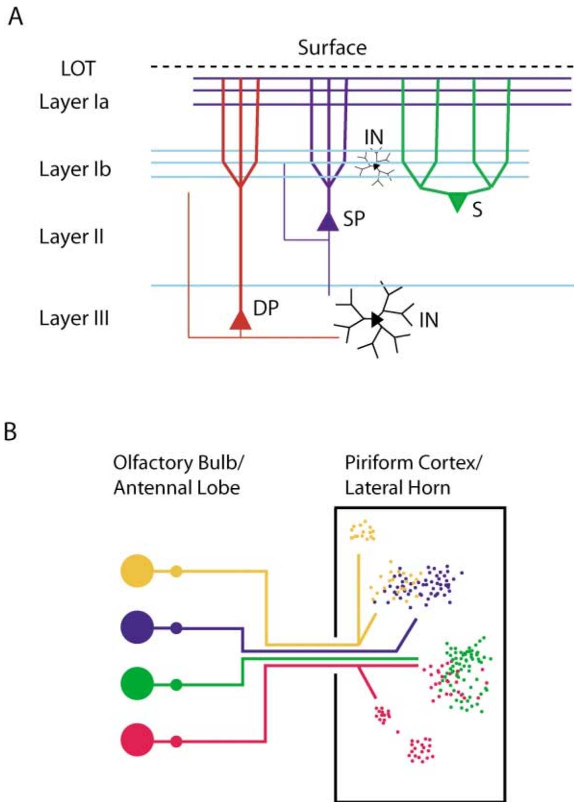

Figure 3. Neurons, Connections, and Terminal Fields in the Piriform Cortex/Lateral Horn

(A) Three-layered structure of the mammalian piriform cortex, with connections from the M/T neurons via the lateral olfactory tract (LOT; dark blue) to the semilunar cells (S), which have apical but no basal dendrites, superficial pyramidal cells (SP), and the deep pyramidal cells (DP), along with several types of interneurons (IN) and associational fibers (light blue). Inputs from associational fibers (Figure 3A, light blue) linking olfactory cortical areas terminate on the apical dendrites in layer 1b and on the basal dendrites in layer III. Adapted from Wilson (2001a), with permission.

(B) M/T cells and PNs from each individual glomerulus extend their axons into clustered and overlapping terminal fields within the piriform cortex of mammals and the lateral horn of *Drosophila*, creating a stereotyped map of the glomerular input. Adapted from Zou et al. (2001), with permission.

in which there exists strong feedback from the cortex onto the thalamic relay stations, there exists major feedback to the olfactory bulb from the piriform cortex (Haberly, 1998). This feedback may help refine the information processing in the olfactory bulb and, in turn, influence the nature of the information presented to the piriform cortex.

Several different subregions of the amygdala receive direct projections from the M/T neurons of the olfactory bulb. In particular, the nucleus of the lateral olfactory tract, periamygdaloid cortex, anterior cortical nucleus, and the medial amygdaloid nucleus regions receive direct projections from the main olfactory bulb as well as indirect projections via the piriform cortex and the lateral entorhinal cortex. Notably missing from these target areas is the basolateral amygdaloid nucleus, which is widely implicated in different types of fear conditioning (Fanselow and LeDoux, 1999). However, this nucleus receives information from the piriform cortex, perirhinal cortex, and other areas of the amygdala that are primary targets of the main olfactory bulb (Cousens and Otto, 1998; Schettino and Otto, 2001).

Other areas of the mammalian brain that receive projections from the olfactory bulb include the perirhinal cortex and the entorhinal cortex and therefore contain 3° olfactory neurons (Carmichael et al., 1994; Haberly, 1998; Zou et al., 2001). In *Drosophila*, olfactory information is also presented to an area known as the inferior lateral protocerebrum (Ito et al., 1998) along with the mushroom bodies and lateral horn (Figure 1). However, knowledge of connections at this level is still evolving in both mammals and *Drosophila*, and there are differences in the projection patterns among representatives of the class Mammalia (Insausti et al., 2002).

From the perspective of hierarchy, the defined 3° neurons of *Drosophila*—the mushroom body neurons and the lateral horn neurons—are therefore the equivalents of neurons in the mammalian amygdala, perirhinal cortex, entorhinal cortex, and/or the piriform cortex. The 3° neurons in both the amygdala and piriform cortex are known to project to the entorhinal cortex, which, in turn, sends information into the dentate gyrus/hippocampal complex. The role of the hippocampus in olfactory learning or other types of learning is beyond the scope of this review. Three different areas of the *Drosophila* brain are putative output regions of the mushroom bodies, including the superior medial protocerebrum, inferior medial protocerebrum, and superior lateral protocerebrum, but the neurons in these areas that potentially receive information are still undefined (Ito et al., 1998). In addition, there exist major pathways for feedback in the mammalian olfactory nervous system onto neurons one step lower in the hierarchy (red arrows in Figure 1). No feedback neurons have yet been conclusively identified from the mushroom bodies or lateral horn in insects to the antennal lobe, although anatomical studies of the antennal lobe suggest that these may exist (Hildebrand and Shepherd, 1997).

The neuroanatomy thus suggests that distinct odors are represented first, in part, by the stimulation of distinct sets of ORNs; second, by the activity of specific M/T-PNs whose identity can be uncovered from their glomerular projections (Hildebrand and Shepherd, 1997; Gao et al., 2000; Vosshall et al., 2000; Ng et al., 2002; Wang et al., 2003); and third, by a distinct set of synaptic fields activated in the 3° olfactory neurons (Marin et al., 2002; Wong et al., 2002; Tanaka et al., 2004).

Representation of Olfactory Cues in the Olfactory Nervous System

The stimulation of *Drosophila* with odors produces a variety of response dynamics in the action potentials generated in their ~1300 ORNs (de Bruyne et al., 1999, 2001; Lessing and Carlson, 1999). Before stimulation, individual ORNs exhibit spontaneous action potentials at frequencies of 3 to 30 spikes/s (Figure 4). During stimulation, the maximum frequency can exceed 200 spikes/s, but this is dependent on odor concentration. In addition, an individual ORN can be excited by some odors and inhibited by others, and an individual odor can excite some neurons but inhibit others. Some excitatory

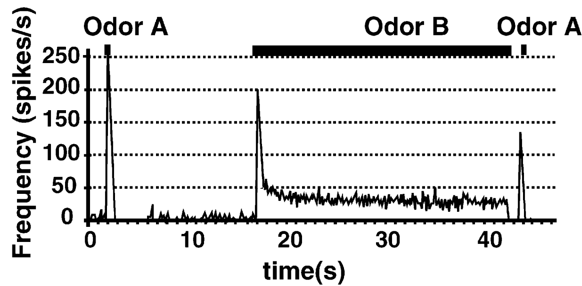

Figure 4. Spike Response Dynamics and Spike Adaptation of *Drosophila* ORNs

Odor stimulation (Odor B) of *Drosophila* ORNs can produce a rapid increase in the spike frequency over the first few seconds of stimulation, followed by spike frequency adaptation. Spontaneous spike firing is illustrated for the periods before stimulation with Odor B and Odor A. The prolonged stimulation with Odor B also produces cross-adaptation, measured as the difference in spike frequency with Odor A before stimulation with Odor B compared to the spike frequency after stimulation with Odor B. Adapted from de Bruyne et al. (1999), with permission.

responses are notably prolonged, persisting well past the termination of odor delivery, whereas others terminate coincidently with odor removal. Moreover, the spike frequency generated in an ORN adapts with prolonged olfactory stimulation, and prolonged exposure to one odor can produce cross-adaptation to another odor (Figure 4). Several of these properties, including response valence, odorant tuning, level of spontaneous spiking activity, and poststimulus spiking frequency, are determined by the olfactory receptor that is expressed in the ORN and not by ORN environment, as shown by ectopically expressing a panel of olfactory receptors individually in the same ORN devoid of its own receptor and recording the response properties (Hallem et al., 2004).

The response properties of mammalian ORNs have also been detailed by single-unit recording and, in general, are very similar to the insect ORNs represented by those from *Drosophila* (Duchamp-Viret et al., 1999). The neurons are broadly tuned, responding with altered spike frequency to many different odors, display spontaneous activity, can be excited or inhibited by different odors, have responses that either coterminate with or extend beyond the odor stimulus, and show spike adaptation during the odor stimulation. It is likely that these response properties reflect the initial neural code for different odors, with the spike frequency and the number of ORNs that are activated perhaps encoding the intensity of an olfactory stimulus; the unique ensemble of ORNs that are activated by any particular odor, spike train dynamics, and response valence perhaps encoding the quality of an olfactory stimulus; and the distribution of spikes over time encoding the presence or absence of an olfactory stimulus. Some of these response properties are also potential substrates for modification by olfactory learning, perhaps through feedback neurons in the olfactory bulb.

The spike frequency information from activated ORNs arriving at the antennal lobe/olfactory bulb initiates a distributed response among the 2° neurons, since the ORNs that express the same olfactory receptor converge on the same glomerulus, and most odors stimulate multiple olfactory receptors. The activation of an ensemble of ORNs expressing a few different olfactory receptors capable of binding to the odorant molecules therefore initiates activity in the glomeruli that house the axon terminals of the ORNs along with dendritic processes of the postsynaptic, 2° neurons (Figure 2). There is also tremendous convergence of information onto the 2° olfactory neurons, which needs to be considered when formulating models about how the brain represents odors and how learning modifies these representations. For instance, the ~1300 ORN axons of *Drosophila* converge on ~43 glomeruli (Lessing and Carlson, 1999), and the several million ORN axons of the mouse converge on ~1800 glomeruli (Hildebrand and Shepherd, 1997; Mori et al., 1999; Mombaerts, 2001). Functional imaging experiments have allowed for the visualization of odor-stimulated patterns of activity in 2° olfactory neurons within the antennal lobe/olfactory bulb. Calcium dyes, voltage-sensitive dyes, transgenically supplied fluorescent proteins, and intrinsic optical signals have been used to visualize odor-specific patterns of glomerulus activation in *Drosophila*, honeybee, zebrafish, salamander, and rat (Friedrich and Korsching, 1997; Joerges et al., 1997; Rubin and Katz, 1999; Kauer and White, 2001; Ng et al., 2002; Wang et al., 2003).

Electrophysiological recordings from the 2° M/T neurons in the mammalian olfactory bulb have revealed several important features relevant to how odors are represented (Mori et al., 1999). Single-unit recordings from individual M/T neurons have been used to probe the molecular receptive range of the M/T neurons, by recording responses from the neurons while challenging the animal with odor molecules that share characteristic molecular features. Thus, M/T neurons often exhibit excitatory spike responses to odors with similar molecular features and can be characterized according to their molecular receptive range. However, M/T neurons can also be inhibited by odorant molecules that have structural features related to those odorants that stimulate it. This is due to the lateral inhibition mediated by the granule cells, since pharmacologically blocking inhibitory synapses can reduce this lateral inhibition. In addition, any given odor can stimulate many different ORNs, leading to the excitation of multiple sets of M/T neurons. Thus, the ensemble of M/T neurons that are activated in part represents the quality of an odor at the level of the olfactory bulb. Since many 2° neurons are excited simultaneously, this produces oscillations in the field potential that can be detected by optical imaging (Spors and Grinvald, 2002) or electrophysiological recording. Indeed, if one records from pairs of M/T neurons during the process of olfactory stimulation, about one-fourth of the neurons show synchronized discharges (Figure 5; Kashiwadani et al., 1999). These oscillations are produced, in part, from the inhibitory circuits built into the olfactory bulb/antennal lobe (Figure 2); the available data suggest that the degree of dendrodendritic and long-range inhibition determines, in part, the probability that two M/T neurons will be synchronized. Thus, the intraglomerular and interglomerular interactions that occur within the olfactory bulb serve to bind together the spiking activity of M/T neurons from distinct glomeruli. The tuning of a M/T neuron's molecular receptive range

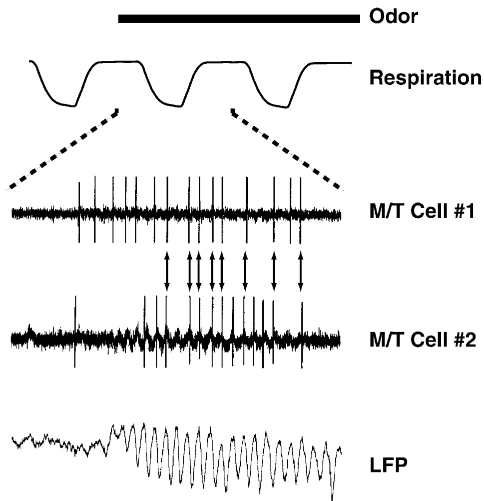

Figure 5. M/T Spiking Activity Is Phase Locked to the Local Field Potential

Odor application (solid bar) produces synchronous spiking activity in M/T neurons during the inhalation portion of the respiratory cycle (downward deflections in the respiration trace). This synchronous firing is phase locked to the local field potential. Data adapted from Kashiwadani et al. (1999) for illustration purposes, with permission.

and the inhibitory interactions within the olfactory bulb, which influence the ensemble of responding M/T neurons and their degree of synchronous activity, offer attractive sites for potential plasticity underlying different forms of olfactory learning.

Similar odor-driven local field potentials and spiking activity have been studied in several insects, including locusts, honeybee, *Manduca*, and *Drosophila* (Laurent and Davidowitz, 1994; MacLeod and Laurent, 1996; Stopfer et al., 1997; MacLeod et al., 1998; Laurent, 2002). PNs and LNs of the antennal lobe of the locust and honeybee exhibit synchronized oscillations at 20–30 Hz (Laurent and Davidowitz, 1994; MacLeod and Laurent, 1996; Stopfer et al., 1997; MacLeod et al., 1998; Laurent, 2002). Although the local field potential oscillations are sustained over the course of the olfactory stimulus and beyond, any individual PN may participate in the population oscillation for only short periods, in a manner that is both neuron and odor specific. The local field oscillations are shaped by inhibitory LNs in the antennal lobe, since the local field potential oscillations can be blocked by the injection of picrotoxin (MacLeod and Laurent, 1996). Thus, the olfactory response that is read at the level of populations of PNs includes an ensemble of oscillating PNs, with any individual member of the ensemble joining the group response for some cycles but not others (for an alternate interpretation, see Christensen et al., 2003).

In the locust and *Drosophila* (Perez-Orive et al., 2002; Wilson et al., 2004), PNs exhibit considerable spontaneous activity. They are broadly tuned to odors, with ~60% of the odors eliciting an excitatory response in any given PN, and with many responses enduring well beyond the time window of odor presentation. LNs are also very broadly tuned, responding to every stimulus presented, as expected from their widespread arborization into most if not all glomeruli. PN spiking in response to odor can be both excitatory and inhibitory, but the responses are stereotyped between animals if recording from PNs that innervate the same glomerulus. Most importantly, the tuning profile for one particular PN proved to be substantially broader than that of its presynaptic ORN, suggesting that the PN responses are not formed solely from the ORN input, but from other sources as well (Wilson et al., 2004). This is an important point, since there is much written about "glomerular responses," as if a glomerulus were a unit of activity or implying a homogenous response of all neurons that innervate the glomerulus, when, in fact, the individualized response patterns of neurons innervating each glomerulus need to be considered. However, the expanded tuning of PNs relative to the presynaptic partners was demonstrated with only one ORN-PN pair. Whether this generalizes to all synaptic pairs remains unknown.

The electrophysiological characterization of M/T neurons and PNs has therefore spawned the idea that the neural representation of odors at the level of the 2° olfactory neurons contains both components of time and space. It is not only the ensemble of the M/T-PNs that are activated by any given odor that is important for its representation; the temporal response properties of the M/T-PNs that comprise the responding ensemble are also part of the representation (Laurent et al., 1998).

Field recordings from the mushroom bodies of insects have also revealed subthreshold, 20–30 Hz oscillations that are produced by input from the antennal lobe (Laurent et al., 1998). Odor representations, as viewed through the window of spiking activity, have also been compared between the antennal lobe PNs and the mushroom body cells (Perez-Orive et al., 2002). In contrast to the high probability of response to olfactory stimulation exhibited by PNs, the mushroom body neurons are relatively quiescent. Thus, the transfer of information is inhibited in some manner between these two olfactory neurons. A systematic study of the possible mechanisms led to the discovery that the mushroom body neurons are inhibited from responding to PN stimulation, in an odor-stimulated manner, by GABAergic neurons in the lateral horn that synapse onto the mushroom body neurons (Perez-Orive et al., 2002). These inhibitory neurons are also stimulated by the PNs with every odor tested and, in turn, inhibit the response of mushroom body neurons. Thus, the 3° olfactory neurons represented by mushroom bodies have an exceptionally large constraint for responding to odors, imposed by feed-forward inhibitory neurons of the lateral horn. Consistent with these physiological experiments demonstrating this constraint, optical imaging experiments of odor-induced calcium transients in mushroom body neurons have shown that different odors activate small sets of mushroom body neurons, stereotyped in position between animals (Wang et al., 2004).

Why is there a strikingly large constraint imposed on mushroom body neurons? Theoretical considerations (Olshausen and Field, 2004) and intuition suggest that associative learning may be facilitated when sensory information is represented by relatively small networks of neurons compared to large, distributed networks. Associations formed with small network representations, in principle, should be easier to form, easier to retrieve,

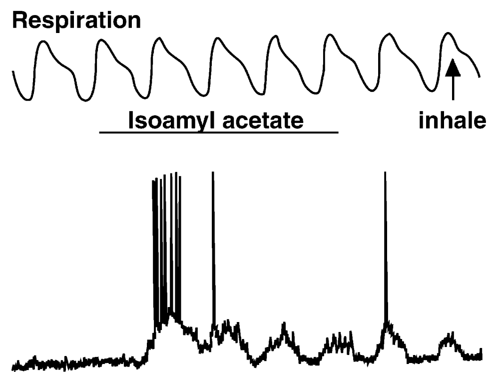

Figure 6. Spiking Activity and Adaptation of Neurons in the Piriform Cortex

Spiking activity recorded from neurons in layers II and III of the piriform cortex exhibit bursts of spiking activity that are entrained with the inhalation phase of the respiratory cycle. Spike frequency diminishes over the time that the odor is applied. Subthreshold oscillations of 30–60 Hz can be observed to ride on top of the depolarization entrained with inhalation. Adapted from Wilson (2001a), with permission.

and subject to fewer errors. Thus, the small sets of mushroom body neurons that represent any given odor may be the preferred substrate for associative learning, over the large sets of PNs. An alternative and equally attractive model envisions the inhibitory constraint as a network-level memory suppressor system and therefore a possible site for learning-induced modifications. Learning could inhibit the inhibition that exists in the naive state, modifying the output of the feed-forward inhibitory neurons or the ability of the mushroom body neurons to respond to inhibitory input. Thus, more mushroom body neurons would be stimulated by a conditioned odor after learning or, alternatively, the naive set would respond more robustly.

As in the insect mushroom bodies, olfactory stimulation induces a strong oscillation of 40–60 Hz in the field potential of the mammalian piriform cortex (Haberly, 1998). But unlike the insect mushroom bodies, the piriform cortex neurons exhibit rather robust responses to many different odors (Schoenbaum and Eichenbaum, 1995; Haberly, 1998; Wilson, 1998, 2000a, 2001a). The responses to odors are generally short in latency and usually excitatory (Figure 6; Wilson, 1998, 2001a), with spiking activity that is entrained with the respiratory cycle. Prolonged stimulation produces a rapid decrease in spike frequency (Figure 6) or spike frequency adaptation. Some layer II/III neurons exhibit lateral specificity for stimulation: some neurons respond only to ipsilateral stimulation, some respond only to contralateral stimulation, some respond to either ipsilateral or contralateral stimulation, and some require simultaneous ipsilateral and contralateral stimulation (Wilson, 1997). The mechanisms for the spatial filters on these stimulation properties are unknown.

There are no studies that have detailed the response to odor cues by other 3° olfactory neurons in the entorhinal cortex, the perirhinal cortex, and the amygdala in ways that parallel the studies described above.

Learning about Olfactory Cues by the Olfactory Nervous System

1° Olfactory Neurons

Current views envision the ORNs as transducers of airborne olfactory information into a neural signal without the capacity for significant associative plasticity. These neurons do adapt to olfactory stimulation and therefore may produce nonassociative behavioral adaptation. However, as discussed above, there exist feedback and modulatory neurons that project to the antennal lobe/olfactory bulb, and it is conceivable that learning might modulate the presynaptic release of neurotransmitters from the ORN via these modulatory neurons. This possibility as a mechanism for olfactory learning has not yet been explored.

2° Olfactory Neurons

Olfactory Perceptual Learning. Perceptual learning is a form of implicit memory that can be defined as an increased sensitivity to stimulus parameters that improves perceptual acuity due to experience (Gilbert et al., 2001). Although the mechanisms for perceptual learning are better understood for visual stimuli, several of the response properties of olfactory system neurons that change with repeated exposure to an odor appear to improve olfactory discrimination and thus constitute a neural correlate of olfactory perceptual learning.

One property discovered to change with exposure is the molecular receptive range (or field) of M/T neurons (Fletcher and Wilson, 2003). The spiking activity of rat M/T neurons was measured after a short exposure of the animal to a series of eight different ethyl ester odorants differing only in the length of the carbon chain backbone. Many M/T neurons responded with robust spiking activity to an ester with four carbons in the backbone chain and with less activity to ethyl esters with one fewer or one more carbon atom in the backbone. The molecular receptive range—the length range of carbon chain backbone for ethyl esters capable of producing spiking activity—was measured using this complete series of ethyl esters. In general, the receptive range was found to be about three carbon atoms in length. Remarkably, however, the optimal receptive range was found to undergo a slow change if the animal received a long exposure to an ethyl ester odorant just outside of the neuron's receptive range, differing from the optimum length by two carbon atoms. Immediately after the long exposure, the receptive range was not changed, although the neuron exhibited spike frequency attenuation (Figure 7). When probed 1 hr later, however, the receptive range was reset, shifting toward the nonoptimal odor after a long exposure to this odor (Figure 7). Thus, experience with the odor shifted the M/T receptive range, potentially enhancing discrimination. The mechanism underlying this shift in receptive range is unknown but presumably involves circuit changes in the olfactory bulb. Although it is attractive that such changes in M/T neuron receptive range are employed for olfactory perceptual learning, experiments to perturb the receptive range in the context of a behaving animal are needed to solidify the relationship.

A second type of sensory plasticity that may underlie perceptual learning was discovered in the antennal lobe of the locust (Stopfer and Laurent, 1999). The coherent spiking activity of ensembles of PNs described above

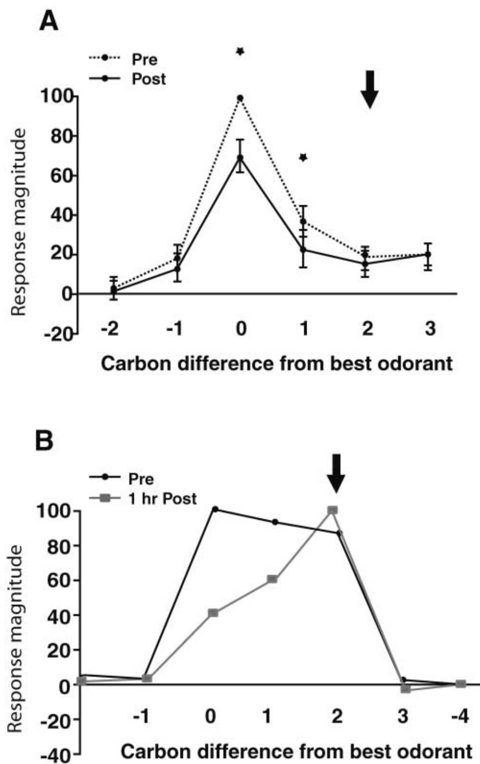

Figure 7. Exposure to an Odor Shifts the Molecular Receptive Range of M/T Neurons

The receptive range of a M/T neuron measured as spike frequency with exposure to a series of ethyl ester odorants differing only in the length of the carbon chain backbone. The response of the neuron has an optimum but also responds to ethyl ester odorants with one fewer or one more carbon than the optimum. (A) Prolonged exposure to an ethyl ester odorant (arrow) outside of the naive receptive range has no effect on the receptive range profile when tested immediately after exposure but produces spike adaptation. (B) Prolonged exposure to an ethyl ester odorant (arrow) outside of the naive receptive range skews the receptive range profile toward the practiced odor when tested 1 hr after exposure. Modified from Fletcher and Wilson (2003), with permission.

actually develops over the course of repeated odor exposures. During this evolution, the spiking activity of PNs decreases, and the subthreshold oscillation at 20 Hz develops in the local field potential, so that the PN spikes gradually become phase locked with the oscillation in the local field potential, increasing their coherence. These dynamic changes reach a stable state after about 8×1 s odor presentations and persist for about 12 min, before the system is reset. The sequential exposure to an odor that produces an increasingly coherent representation of that odor offers an intuitively attractive way for the antennal lobe/olfactory bulb to develop better stimulus discrimination. Behavioral and other electrophysiological results support the idea that coherent oscillatory activity in the antennal lobe is required for the correct identification and discrimination of similar odorants (Stopfer et al., 1997). Honeybees fail to discriminate an odorant (1-hexanol) trained with a sucrose reward from an odorant of similar structure (1-octanol) if the synchrony of oscillations is disrupted by injection of picrotoxin into the antennal lobe to block $GABA_A$ receptors. Nevertheless, they retain the ability to discriminate odorants of dissimilar structure. Picrotoxin injection into the antennal lobe of locusts also causes neurons downstream of the PNs to misinterpret the sensory stimulus (MacLeod et al., 1998). In extreme cases, picrotoxin injection caused the downstream neurons to respond to odors for which they were previously unresponsive. Moreover, a mouse knockout that increases the amplitude of olfactory bulb oscillations has been reported to display improved olfactory discrimination in some tests (Nusser et al., 2001). These observations combine to suggest that repeated exposure to an odor initiates the evolution of a neural response assembly that allows for improved discrimination.

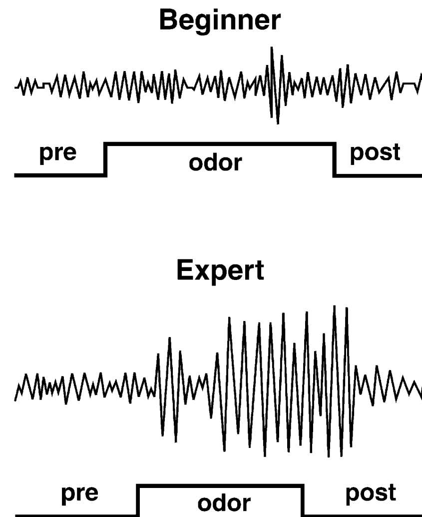

Figure 8. Increased β Oscillations in Olfactory Bulb Field Potentials in Rats in the Early and Late Phases of Operant, Odor-Discriminative Training

The traces illustrate weak β oscillations during odor sampling in animals at the early (beginner) stages of training but robust oscillations at the late (expert) stages of training. Adapted from Ravel et al. (2003), with permission.

Operant Olfactory Conditioning. Several reports have shown that changes occur in the oscillations of local field potentials concurrent with operant olfactory learning (Ravel et al., 2003; Martin et al., 2004). Rats were trained in go/no-go odor discrimination tasks while oscillatory activity in local field potentials in the olfactory bulb was monitored. These tasks extended over a period of 2–3 weeks, so changes observed after this period in highly trained animals must reflect long-term memory. The animals would sample one of two odors from an odor port, one of which signaled the delivery of a positive reinforcer (food or sucrose water); the other signaled the absence of a reinforcer or the delivery of an aversive reinforcer (quinine water). After numerous trials, the animals learned a clear discrimination between the odors, proceeding rapidly to their rewarded destination after sampling the reward-associated odor but hesitating significantly before approaching the anticipated bitter drink or absence of a reward. Oscillatory activity of field potentials in the olfactory bulb exhibited three interesting changes with training. There was an increase in the power of ~25 Hz (β oscillations) oscillations in well-trained animals compared to beginners (Figure 8). In

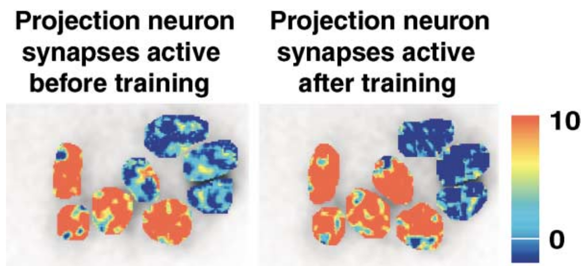

Figure 9. Recruitment of New PN Synapses in the *Drosophila* Antennal Lobe after Classical Conditioning

The activity of PN synapses reported by synapto-pHluorin, a fluorescent and transgenically supplied reporter of synaptic activity, in response to one odor prior to conditioning is shown in the left panel. A color scale indicates the change in fluorescence response in the glomeruli housing the PN terminals upon stimulation with an odor. This panel shows that PNs innervating four of eight glomeruli are stimulated by the odor prior to conditioning. After conditioning (right panel), PNs innervating a fifth glomerulus joined the representation within 3 min after conditioning. No quantitative changes were observed after conditioning in the activity of the four sets of PNs that respond to the odor prior to conditioning. Adapted from Yu et al. (2004), with permission.

addition, the power of oscillations that were induced by the reward-associated odor was stronger than that of those induced by the counterodor. Finally, the amplified ~25 Hz oscillations occurred while the animals sampled the odors but then disappeared before the animals initiated a behavioral response. These observations are consistent with the hypothesis that the oscillations are directly related to odor processing and learning but prompt several important questions. What is the neural basis for the increased oscillatory activity? One possibility is that more neurons are recruited into an ensemble of neurons that respond to the learned odor, thus increasing the power of the oscillations. Alternatively, the neurons that are involved in odor representation may become more precisely synchronized. Which neurons both within and outside of the olfactory bulb contribute to the generation of the increased oscillations? Evidence (Ravel et al., 2003; Martin et al., 2004) suggests that the oscillations are not intrinsic to the olfactory bulb but require the participation of higher brain structures, perhaps including feedback from the piriform or entorhinal cortices (Figure 1). Finally, can the odor-induced oscillations be disrupted in highly trained animals so as to disrupt their learned discrimination?

Classical Conditioning. Several studies have demonstrated that learning-correlated changes in neural activity occur in the antennal lobe of insects after olfactory classical conditioning. In one recent study, Yu et al. (2004) searched for altered synaptic activity among the synapses made by the three types of neurons in the *Drosophila* antennal lobe: the ORNs, the PNs, and the GABAergic LNs (Figure 2). The researchers used different GAL4 transgenes exhibiting cell type specificity to express a novel reporter of synaptic transmission, synapto-pHluorin, at the antennal lobe synapses of each of these classes of neurons. Flies were classically conditioned with odor and electric shock while immobilized under a laser-scanning confocal microscope, and their antennal lobes were scanned to record the fluorescence responses from individual glomeruli (Figure 9). Since PNs are uniglomerular, their intraglomerular (Figure 2) synaptic responses could be monitored before and after conditioning by examining the fluorescence from individual glomeruli. Possible changes in the ORNs and the LNs were also assayed.

The experiments produced unexpected evidence for the existence of a short-term olfactory memory trace represented as newly recruited synaptic activity in the terminals of the PNs (Yu et al., 2004). Conditioning with both odor and electric shock produced no change in fluorescence relative to the naive response in the terminals of the ORNs. Similarly, no changes in synaptic activity due to conditioning were detected among the terminals of the LNs. The PNs, however, exhibited a robust change with conditioning. Although the odor by itself activated synaptic transmission from PNs innervating four of the glomeruli that were imaged, conditioning produced the activation of PNs innervating a fifth glomerulus (Figure 9). These changes were observed within 3 min after conditioning, and the increased response lasted 7 min before fading back to baseline. Thus, classical conditioning recruited the activity of new PN synapses into the representation of the conditioned odor. The speed at which the new synapses entered the representation suggests that the synapses were present but relatively inactive, with conditioning activating an "on" switch. The alternative, that the synapses were sprouted due to conditioning, is less likely, since new synaptic growth would probably require more than 3 min. Moreover, the pattern of synapse recruitment is dependent on the odor used for conditioning. One set of PNs was recruited with one odor, yet another set was recruited by a second odor. Thus, the evidence suggests that classical conditioning spurs the development of a short-term memory trace in the antennal lobe in the form of newly recruited synaptic activity of PNs.

A potentially related study using the moth *Manduca* with odors and a sucrose reward has also demonstrated that changes in neural activity occur with classical conditioning in the antennal lobe (Daly et al., 2004). Multichannel electrode arrays that were implanted into the antennal lobe of moths were used to detect spiking activity of ensembles of neurons before and after odor-reward conditioning. With successive training trials over a period of 1 hr, an increasing number of responding neurons was detected, such that after ten training trials, the number of neurons responding to the odor increased by 60%. Although the major change with forward pairing of odor and sucrose was the recruitment of initially unresponsive neurons over the course of discriminative conditioning in which both a conditioned stimulus (CS)+ and a CS− odor were used, some neurons actually shifted their response state from being excited by the odor to being inhibited, or vice versa. In addition, the conditioning caused a major temporal restructuring of some responses. More specifically, phases of excitation that occurred with odor prior to conditioning were in some cases lost, gained, or shifted in their latency relative to the odor stimulus. Although the identity of the neurons that were recorded remains unknown, and the mechanism underlying these changes is completely mysterious, the results nevertheless suggest that there are major changes in neural responsiveness in antennal lobe neurons that occur with classical conditioning, the

major change being a recruitment of new neurons into the ensemble of those excited by the conditioned odor.

A third related study utilized classical conditioning of the honeybee with visualization of neural activity in the antennal lobe using calcium indicator dyes (Faber et al., 1999). Differential conditioning to two odors led to an increase in the calcium response in certain glomeruli after conditioning. Glomeruli that exhibited a calcium response to the odor before conditioning increased in their responsiveness after conditioning, and there appeared to be an expansion of responsiveness after conditioning into glomeruli that showed no or modest calcium responses before conditioning. The identity of the neurons that exhibited altered calcium signals due to conditioning remains unknown, but the *Drosophila* results above suggest that these might be the PNs. In addition, a very preliminary report claims that altered responses occur after conditioning in the lip region of the calyx of the mushroom bodies (Faber and Menzel, 2001), a neuropil area that houses the axon terminals from the PNs as well as the dendritic processes of the mushroom bodies. A conditioned response at this location might represent increased calcium influx into the terminals of the PNs.

Altogether, these studies provide compelling evidence for the formation of olfactory memory traces due to classical conditioning in the antennal lobe of insects. The trace is registered in one case as a recruitment of PN synaptic activity into the representation of the conditioned odor; in another, by the recruitment of PN spiking activity into the representation; and in a third, by an increased intensity of the representation measured as calcium influx and perhaps the recruitment of previously unresponsive neurons as well. The mechanisms underlying each change are unknown. Nor are there studies that prove the significance of these changes to behavior. The results remain, therefore, correlative but nonetheless provocative.

3° Olfactory Neurons

Perceptual Learning. Wilson has studied potential electrophysiological correlates in the anterior piriform cortex underlying experience-dependent changes in odor discrimination by the rat. For some experiments (Wilson, 2000a, 2000b), he used single-unit recording from M/T cells in the olfactory bulb and neurons in the anterior piriform cortex to examine odor-evoked spiking in these neurons in response to alkane hydrocarbon or other odorants, after preexposure to either the same (self) or a closely related odorant (cross). M/T neurons proved to be very susceptible to cross-adaptation, detected as a marked decrease in spike rate evoked by one odorant after preexposure to a related one, whereas neurons of the anterior piriform cortex were much more refractory to odorant cross-adaptation. These results suggest that neurons of the anterior piriform cortex are higher-fidelity discriminators of closely related odors. The better discrimination of the anterior piriform cortex neurons may be due to cholinergic input-induced refinement of receptive fields, possibly from cholinergic terminals from the horizontal limb of the diagonal band of Broca, since application of the muscarinic receptor antagonist scopolamine to the piriform cortex neurons produces significant spike frequency cross-adaptation to odors that normally show no cross-adaptation (Wilson, 2001b).

In a test designed to probe the familiarization time required for anterior piriform cortex neurons to develop the physiological correlates of a synthetic receptive field, one presumed substrate of a perceptual learning event, the same procedures were used to examine odor-evoked spiking in these neurons to a mixture of two odors and to one of the component odors after preexposure to the odor mixture (Wilson, 2003). Preexposure of the animal to a mixture of two odors for a 50 s familiarization period caused a marked decrease of odor-evoked spiking in M/T neurons to both the odor mixture and one of the components when tested after the preexposure period. Self-adaptation and cross-adaptation to the mixture and the pure component, respectively, were also observed in anterior piriform cortex neurons after only a 10 s preexposure period, but these neurons regained their responsiveness to the odor mixture and pure component if the preexposure was extended to 50 s. One interpretation of these results is that the longer preexposure to the mixture provides sufficient experience for overlapping receptive fields of the individual components in the anterior piriform cortex to spawn the development of a synthetic receptive field, so as to increase the perception of the mixture, whereas such plasticity is not available at the level of the olfactory bulb with the same level of experience. Although it is most parsimonious to infer that the relevant changes occurred within the anterior piriform cortex neurons, it is possible that the changes are actually presynaptic to these neurons and therefore occur within the M/T neurons or are provided by neurons extrinsic to the olfactory nervous system that provide input to the piriform cortex neurons.

Operant Conditioning. Studies have also revealed that the piriform cortex changes in odor response properties after operant conditioning. Litaudon et al. (1997) implanted electrodes into the olfactory bulb of rats and discriminatively conditioned the rats, pairing the stimulation of one electrode with sucrose water for a thirsty rat and the other with quinine water. Rats learn this discriminative task over a period of a few days, eventually selecting the sucrose water when cued by the appropriate stimulation of the olfactory bulb and avoiding the quinine water when cued by stimulation from the other electrode. The response properties of the piriform cortex measured optically with a voltage-sensitive dye were then determined in discriminatively trained and control rats. Several changes were observed in the optical signal from the trained rats versus the controls upon bulbar stimulation, including an increase in the probability of occurrence of a late response component in piriform cortex and a broader spread of this component over the posterior cortex. No differences were observed in responses associated with the positive reinforcer and those associated with the negative reinforcer. In a second study (Mouly et al., 2001), the evoked field potential was monitored in the trained rats. Increases in the magnitude of the evoked field potential were detected in animals having learned this discriminative task relative to mock control animals in the lateral entorhinal cortex (LEC) with the sucrose-associated stimulation after four daily sessions of training. This increased signal was maintained to a retest at 20 days later. In addition, the posterior piriform cortex but not the anterior piriform cortex also exhibited an increase at 20 days in awake

animals but not at the first postlearning test. No significant changes in the magnitude of the evoked field potentials were detected in awake trained animals relative to the controls in these areas in response to stimulation by the electrode paired with quinine. Other studies have also revealed changes in components of the evoked field potentials in numerous areas of the olfactory nervous system (Mouly and Gervais, 2002). These observations are supportive of plastic changes in the piriform cortex and other areas of the olfactory nervous system in response to operant conditioning but offer no insight into the identity of the cell types involved in the changes, the nature of the changes at the cellular level, or the physiological mechanisms for the changes.

A series of experimental studies (Schoenbaum et al., 1998, 1999, 2003) have evaluated the role of the basolateral amygdala (and the orbitofrontal complex) in discriminative olfactory conditioning. The orbitofrontal/basolateral amygdala system, which consists of reciprocal connections from the basolateral amygdala to the orbitofrontal cortex, has been implicated in the ability to learn the motivational significance of cues and to make appropriate decisions from that significance. Unit recordings of rat neurons in the basolateral amygdala show that these neurons can develop an increased selectivity for responding to specific odors that predict the delivery of an appetitive (sucrose) substance, for instance, over odors that predict the delivery of an aversive (quinine) substance. For example, some basolateral amygdala neurons increase their firing rate over many training trials in response to an odor cue that predicts the delivery of sucrose. Interestingly, that odor selectivity in response can be reversed with reversal training, in which a second odor becomes paired with the sucrose reward. These responses occur while the animal is sampling the odor, making it clear that neurons of the basolateral amygdala can respond to specific odors after learning. However, animals with a lesioned basolateral amygdala still learn to discriminate the odors paired with appetitive or aversive substances at a rate indistinguishable from the controls, indicating that the odor representations made by these neurons are dispensable for learning. Nevertheless, the lesioned animals fail to develop the decreased latency after odor sampling to taste the anticipated reward or the increased latency after odor sampling to taste the aversive stimulus. These latency changes are characteristic of control animals. Thus, the combined evidence indicates that the odor representations in the amygdala are not required to support olfactory discriminative operant conditioning but are necessary to support the associated motivational component of the odor cue.

Classical Conditioning. An olfactory-based, fear conditioning paradigm modeled after the more typical auditory cue/contextual fear conditioning paradigms has been developed and used to probe the contribution of various 3° olfactory neurons to olfactory fear conditioning through lesioning studies (Otto et al., 2000). In this paradigm, rats are presented with 20 s of odor and a 2 s footshock that coterminates with the odor presentation. A training session of six odor/shock pairings separated by a 4 min intertrial interval leads to strong freezing behavior in response to the odor alone 24 hr after conditioning. Lesions that were induced prior to training in the basolateral amygdala, which receives indirect projections from the main olfactory bulb via the corticomedial amygdala group and the perirhinal cortex (Figure 1), essentially eliminated the conditioned fear response to the odor. Lesions to the basolateral amygdala that were induced either 1 or 15 days after olfactory fear conditioning also eliminated conditioned responses tested 1 week after lesioning. These results indicate that the basolateral amygdala is absolutely required and has a sustained role in the formation and expression of olfactory fear memory. Lesions to the perirhinal cortex, which also provides input to the basolateral amygdala from the olfactory bulb, impair but do not eliminate olfactory fear conditioning. Thus, the perirhinal cortex may provide some but not all of the input necessary for the function of the basolateral amygdala in olfactory fear conditioning.

Neurons of the basolateral amygdala also display neural correlates of an integration event of an odor with an aversive stimulus (Rosenkranz and Grace, 2002). A series of footshocks to an anesthetized rat produces in these neurons a depolarizing response that can lead to a series of action potentials depending on the stimulus intensity. Stimulation with odors produces a weak or no depolarizing response in these neurons. Odor and footshock pairing, however, increases the initial response to odor or causes the appearance of a depolarizing response in those neurons that initially had no response to the odor by itself. These data demonstrate that amygdala neurons receive both odor CS information and electric shock-unconditioned stimulus (US) information, and they can integrate these into an increased response to the odor CS. The increased depolarization with odor CS after training was correlated with an increased excitability of these neurons, which, along with the increased depolarization with stimulation from the odor CS, was blocked by the drug haloperidol, which can function as a dopamine receptor antagonist. Although haloperidol has other pharmacological mechanisms, these observations are consistent with the possibility that the enhanced depolarizing responses are mediated by dopamine receptor activation and that this may be necessary for behavioral conditioning. Dopamine agonists by themselves had no effect on the postsynaptic depolarization responses to odors. Therefore, it appears that a fast, excitatory stimulation of these neurons from a US pathway conveying the footshock, coupled with odor-induced depolarizations in the presence of dopamine, can alter the excitable state of these neurons to encode the odor-footshock association.

A general note of caution is appropriate regarding several of the aforementioned studies of odor and learning-correlated response properties of 2° and 3° mammalian olfactory neurons. These studies employed anesthetized animals to simplify the in vivo recording procedures. However, anesthesia can change, at minimum, the sensitivity of neurons to stimulation, and it could have broader effects on neuronal activity. Thus, there is a need to utilize awake, behaving animals in future studies to establish with greater confidence that observations made are not a consequence of the anesthetic state.

In a paper that beautifully illustrates the wonderful techniques available with *Drosophila* for dissecting olfactory memory, even when addressing issues at the

systems level, M. Schwaerzel, M. Heisenberg, and colleagues have probed the biogenic amine requirement for both appetitive (sucrose) and aversive (shock) classical conditioning with odors (Schwaerzel et al., 2003). They employed a discriminative olfactory conditioning paradigm that uses the same odors paired with either sucrose or electric shock and showed that *rutabaga* mutants (see below) are impaired in both appetitive and aversive olfactory conditioning but that these impairments can be rescued by expressing a wild-type *rutabaga* transgene with a defined promoter in approximately 700 mushroom body neurons. This demonstrated that the requirement for *rutabaga* activity maps to the same set of neurons for both appetitive and aversive olfactory conditioning. They then blocked the synaptic output of these same neurons by expressing with the same promoter a transgene product that disrupts synaptic transmission at elevated temperatures (Shibire^{ts}) and showed that this block affects memory retrieval of appetitive conditioning, just as prior reports had shown that this blocks memory retrieval of aversive conditioning. Finally, they demonstrated that a mutant in tyramine-β-hydroxylase, which is required for the biosynthesis of octopamine, impairs appetitive conditioning but not aversive conditioning and that this impairment can be rescued by feeding the mutant flies octopamine just prior to but not after training, indicating that octopamine is required at the time of acquisition for normal appetitive learning. Conversely, they used a defined promoter (tyrosine hydroxylase-GAL4) that is expressed in dopaminergic neurons to drive UAS-Shibire^{ts} at restrictive temperatures to show that dopamine is required at the time of acquisition for aversive but not appetitive conditioning. These data, therefore, strongly suggest that dopaminergic innervation of 700 mushroom body neurons is required at the time of acquisition for aversive conditioning and that octopaminergic innervation of the same 700 mushroom body neurons is required at the time of acquisition for appetitive conditioning, distinguishing the requirement for the two different biogenic amines for the two types of olfactory learning. Dopamine (Han et al., 1996; Kim et al., 2003) and octopamine (Han et al., 1998) receptors that stimulate adenylyl cyclase activity have previously been found to be expressed primarily on mushroom body axons, and the biogenic amines presumably signal to the mushroom bodies through these receptors (Figure 10). But if the same 700 neurons mediate both aversive and appetitive olfactory conditioning, and they both do so by activating adenylyl cyclase, how is it that the valence of the US remains unconfused? There are three possibilities. One is that the dopaminergic and octopaminergic pathways are the US pathways and that the response to the biogenic amines is compartmentalized within the neurons in some fashion, so that different signaling ensues. Alternatively, although some or all of the 700 neurons presumably receive CS information, it is possible that only some receive dopaminergic input and others receive octopaminergic input, so that the US pathways (carried by the biogenic amine neurons) remain separate. The latter alternative is the solution that photoreceptor neurons and ORNs have found to maintain stimulus specificity. Although they utilize the same intracellular signaling pathways, they simply express different receptors on their surfaces, keeping different types of sensory information distinct. A third possibility is that the shock US and the sucrose US are presented to the mushroom body neurons through fast, excitatory receptors on different sets of the 700 mushroom body neurons. The biogenic amines are required as well, just as dopamine is required for establishing an odor/footshock memory trace in the basolateral amygdala neurons described above. Electric shocks delivered to the abdomen of the fly do produce rapid excitation and synaptic release from antennal lobe PNs but have no effect on ORNs or LNs (Yu et al., 2004). This must be mediated by fast excitation rather than neuromodulation.

Little information is available about potential memory traces in other 3° olfactory neurons. Studies of the perirhinal cortex for the processing of visual information in nonhuman primates suggest that the cortex participates in objection recognition and memory by binding together the various attributes of an object, including its smell and texture, into a unified representation (Murray and Richmond, 2001; Holscher and Rolls, 2002).

Molecular Genetics and Biochemistry of Olfactory Memory Formation in the Olfactory Nervous System

Molecular genetic analysis of olfactory memory has been pursued so far only with *Drosophila*. Several recent reviews have summarized the progress from different perspectives (Davis, 1996; Roman and Davis, 2001; Waddell and Quinn, 2001; Dubnau et al., 2003b; Heisenberg, 2003). The discussion below is intended to summarize and update these review articles, emphasizing the newer developments over the last few years.

A genetic technique that provides for the identification of olfactory memory mutants with bias toward preferential expression within the hierarchical organization of the olfactory nervous system (Figure 1) is enhancer detection (Davis, 1993). This technique utilizes a transposable element to disrupt genes randomly by insertion, but the transposable element also contains a reporter gene whose expression is influenced by enhancers in the genome near the insertion site. Screens employing enhancer detection have identified several genes required for olfactory learning that are expressed primarily in the mushroom bodies. These mutants, along with those isolated in screens based on behavioral screening (Waddell and Quinn, 2001; Dubnau et al., 2003b), have provided key insights into the molecular biology and biochemistry of olfactory memory through the identification of disrupted genes in various mutants. In addition, most of the mutants that were isolated from behavioral screening with but one notable exception were subsequently discovered to have enhanced expression in the mushroom body neurons. These results, together with disruption experiments for ablating (de Belle and Heisenberg, 1994), disrupting the physiology (Connolly et al., 1996), or blocking synaptic activity (Dubnau et al., 2001; McGuire et al., 2001) of the mushroom body neurons have provided compelling evidence for the hypothesis that mushroom body neurons comprise a principle site for the formation of olfactory memories (Davis, 1993).

An updated version of the cellular model (Davis, 1993)

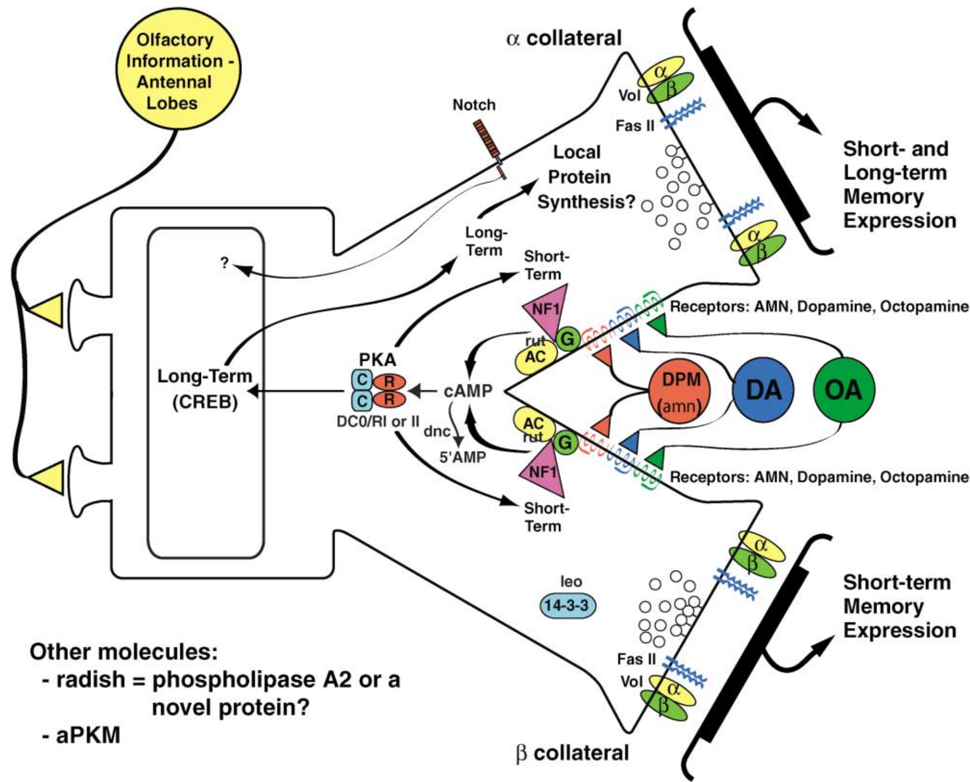

Figure 10. Molecular and Cellular Model for Olfactory Learning as Mediated by Mushroom Body Neurons

for mushroom body participation in olfactory learning is illustrated in Figure 10. This shows one of the three types of mushroom body neurons, called an α/β neuron, as 3° in the olfactory nervous system and therefore within the CS pathway when using odors as the CS. Also shown are pathways that converge on the mushroom body neurons that potentially mediate unconditioned stimuli or represent neuromodulatory inputs necessary for learning. These include inputs from dorsal posterior medial (DPM), dopaminergic, and octopaminergic neurons. In *Drosophila*, the most widely used olfactory classical conditioning paradigm utilizes electric shock as the US. The model also shows some of the gene products that are required for olfactory memory, demonstrated by the failure of animals defective in the expression of those genes to form and recall olfactory memories as well as control animals. One central theme illustrated by this model is that the plasticity of mushroom body neurons that underlies olfactory memory requires the cAMP signaling system, evidenced by the fact that mutants or dominant negatives for the genes *dunce* (*dnc*, cAMP phosphodiesterase), *rutabaga* (*rut*, adenylyl cyclase), DC0 (protein kinase A catalytic subunit [PKA]), and cAMP response element binding protein (CREB) are all impaired in olfactory memory. The requirement for the first three of these molecules is in the initial stages of memory formation, whereas CREB is thought to be required for long-term memory. Moreover, the products of the *amnesiac* (*amn*) gene, thought to be neuropeptides that can activate adenylyl cyclase, may modulate *rutabaga* activity through an unknown but G protein-coupled neuropeptide receptor. One recent advance for the role of *rutabaga* was the demonstration that its function is required only in the adult mushroom bodies for normal olfactory learning (McGuire et al., 2003; Mao et al., 2004). This was shown using newly developed technology that provides for the control of transgene expression in time and in space (McGuire et al., 2004). This solved an important conceptual issue. Adenylyl cyclases are known to be involved in brain development, so the possibility always existed that the learning impairment was due to defective brain development rather than to a physiological role for the adenylyl cyclase in memory formation (McGuire et al., 2003; Mao et al., 2004). The protein neurofibromin, the product of the *Drosophila* NF1 gene, also contributes to the cAMP signaling required for olfactory learning. Neurofibromin is required for the normal activity of the *rutabaga*-encoded adenylyl cyclase. Overexpression of a molecule that presumably blocks the function of CREB (CREB blocker) impairs long-term memory formation, presumably through its action on gene expression in the nucleus. In addition, an activating form of CREB was originally reported to enhance memory formation when overexpressed from transgenes in wild-type flies, but a recent report showed these transgenes to be defective, containing a nonsense mutation in the CREB open reading frame (Perazzona et al., 2004). This now raises a major issue about the role, if any, of this form of CREB in *Drosophila* olfactory learning. The cAMP signaling pathway may be stimulated by neuromodulatory inputs from G protein-coupled receptors, including dopamine

D1-like receptors, octopamine receptors, and a neuropeptide receptor (AMN Receptor).

A second theme is that cell adhesion receptors, including members of the integrin family of proteins (*Vol*) and the immunoglobulin superfamily of proteins (*fasII*; Cheng et al., 2001), are required for the formation of normal olfactory memory. These may function through dynamic cell adhesion and de-adhesion to regulate synaptic structure and plasticity and/or through more classical signaling roles. The *leonardo* (*leo*) gene encodes a 14-3-3 protein, but at present its biochemical role in memory formation remains unknown.

There are a few additional molecular players that have emerged recently, but at present it is not known whether they function within the cellular model depicted in Figure 10 or in other areas of the brain. Drier and colleagues (2002) have studied the role of atypical protein kinase M (aPKM), which is a truncated and persistently active isoform of atypical protein kinase C (aPKC). Pharmacological or dominant-negative inhibition of *Drosophila* aPKM impairs 24 hr memory but does not affect initial learning. Most remarkably, the overexpression of the *Drosophila* or mouse aPKM gene enhances memory when tested at one to several days after training, but only if the expression was induced 30–60 min after training. Inducing the gene before training has no effect. Thus, aPKM can produce an enhancement of performance if it is overexpressed just after training, suggesting that the enzyme participates in the maintenance of memories, perhaps through the maintenance of enhanced synaptic transmission.

A behavioral screen for long-term memory mutants conducted in parallel with microarray experiments to select genes with altered expression after spaced training identified several new putative memory mutants whose gene products are known to function in subcellular localization of mRNAs and local translation (Dubnau et al., 2003a). For instance, the *pumilio* gene encodes a protein that functions as a transcript-specific translational repressor; *staufen* and *oskar* encode proteins involved in mRNA translocation in *Drosophila* oocytes. Some of these products do appear to be expressed in mushroom bodies, and although still preliminary, the results are consistent with the proposal that mRNA translocation and local translation in neuritic processes is an important cell biological process underlying long-term plasticity (Martin et al., 1997).

Notch is a well-studied gene originally defined for its roles in cell type specification. The *Notch* protein is a cell surface receptor with a single transmembrane domain whose intracellular domain is cleaved from the protein upon binding ligands. The intracellular domain then enters the nucleus, where it regulates the expression of target genes. Temperature-sensitive mutants for *Notch* trained at the restrictive temperature as well as dominant negatives for *Notch* induced just prior to training have no effect on initial memory formation but impair long-term memory induced by spaced training (Ge et al., 2004; Presente et al., 2004). Moreover, long-term memory was impaired by the relatively specific expression in the mushroom bodies of an RNAi construct designed to interfere with *Notch* expression (Presente et al., 2004). In addition, the inducible overexpression of wild-type *Notch* function in a normal background produces a marked improvement in long-term memory, which was attributed to an increase in protein synthesis-dependent long-term memory (Ge et al., 2004). These results offer evidence that *Notch* participates in normal long-term memory formation mediated by the mushroom bodies and that its abundance is rate limiting for the formation of protein synthesis-dependent long-term memory. However, there remain many unknowns, including the subcellular distribution of the *Notch* protein, the identity of possible ligands, and the mechanism underlying its effects.

Finally, the *radish* gene, which also compromises olfactory memory when mutated, was recently reported by one group of researchers to encode phospholipase-A2 (Chiang et al., 2004). A second group of researchers, however, has claimed that it encodes a novel protein with possible nuclear localization motifs (E. Folkers et al., 2004, 45th Annual *Drosophila* Research Conference, Genetics Society of America, abstract). It is unproductive to begin modeling any cellular functioning of *radish* until the protein product is identified unambiguously.

Two interesting studies that provide clues about the cellular and subcellular localization of long-term olfactory memory in *Drosophila* were recently published (Pascual and Preat, 2001; Isabel et al., 2004). These investigators discovered and studied a new mutant with variable expressivity that causes the mushroom bodies to be malformed. A large fraction of mutant flies are missing the β and β' axon collaterals, respectively, of the two types of mushroom body neurons, α/β and α'/β', but retain the α and α' axon collaterals. A small fraction of the flies are missing the α and α' axon collaterals of the α/β and α'/β' neurons but retain the β and β' axon collaterals. Surprisingly, these mutant flies when trained and tested together perform indistinguishably from the controls in tests of both short- and long-term olfactory memory. However, the tests of those flies missing only the α and α' axon collaterals have no detectable long-term olfactory memory, produced with a spaced training protocol, whereas those mutants missing the β and β' axon collaterals have normal long-term memory. These results, intriguingly, show that the axon collaterals of either the α/β or α'/β' neurons, or both, have distinguishable functions. The collaterals that project dorsally (α and α') are required for long-term memory, but the collaterals that project horizontally (β and β') are not. The most attractive explanation for these results is that neurons postsynaptic to the α/α' axon collaterals are required for the formation, consolidation, or retrieval of long-term memory, while neurons postsynaptic to the β/β' axon collaterals are not. However, flies engineered to have a conditional block of synaptic output from the α/β mushroom body neurons exhibit a significantly reduced but not completely impaired long-term memory, when the conditional block is applied just before testing (Isabel et al., 2004). These data therefore suggest that at least some long-term memory is formed and stored specifically within the α collateral of α/β mushroom body neurons, potentially using synapse-specific biochemical mechanisms. However, since the block of long-term memory performance was not complete, these data leave open the possibility that some of the neural signals employed during retrieval leak from the α axon collateral due to an incomplete block in synaptic transmission, or

that some long-term memory is formed outside of the mushroom bodies and can be retrieved independently of mushroom body synaptic transmission.

Perspectives

The neurophysiological studies of olfactory learning described above have provided insight into the neural correlates of perceptual, operant, and classical conditioning with odors. It seems likely, for instance, that there exist refinements in the population activity of second order neurons that underlie perceptual learning and that processes exist to recruit the activity of second order neurons into the representation of a conditioned odor during operant or classical conditioning. It also appears that higher order neurons, including those of the basolateral amygdala, may be required for some aspects of olfactory operant conditioning, such as the motivational component associated with an odor cue, and they are also an absolute requirement for classical fear conditioning with an odor CS. Thus, olfactory learning is a process distributed among many neurons of the olfactory nervous system. It remains unclear whether this apparent distribution arises from considering different types of olfactory learning, with each odor being represented by a small network of neurons, or whether larger networks are involved in representing information bits about odors, including perhaps the odor's hue, intensity, valence (positive, negative, or neutral), and valence intensity.

A second conclusion gleaned from the combined studies presented above is that the vast majority of what we think we know is descriptive and correlative. This does not mean that the information is incorrect, of course, but there is a pressing need to pursue perturbation experiments to test whether the neural correlates of olfactory learning are really meaningful to the learning processes themselves. This is much easier said than done, especially when studying changes in activity that emerge from populations of neurons. But until appropriate experimental designs are discovered that can provide for this, the neural correlates will remain only that.

Finally, there exists an enormous gap between the molecular biological/genetics approach and perspective of olfactory learning and the neural systems approach and perspective of olfactory learning. This is undoubtedly due, in part, to different biases. One extreme perspective holds that encoding and learning take place in systems of neurons and therefore little can be gained by analyzing single gene mutations, while those that analyze single gene mutations view the complexity of responses observed at the systems level as beyond our current ability to understand them. There are, however, very reasonable approaches to begin to bridge this gap. For instance, it should now be possible to probe how ensembles of projection neurons respond to odors and how these change during learning in the mutants that impair *Drosophila* olfactory learning.

Acknowledgments

Research on olfactory learning in the author's laboratory has been supported by NINDS grant NS19904; the Mathers Charitable Trust; and the R.P. Doherty-Welch Chair in Science. I thank Ms. Lynda Lynch for assistance with illustrations and preparation of the manuscript. I also thank Mitch Deshazer, Xiu Liu, and David Akalal, members of my laboratory, for their comments on the manuscript; as well as my colleagues in the olfactory learning community, Gilles Laurent, Tim Otto, and Donald Wilson.

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