Review

Neural and molecular mechanisms of microcognition in *Limax*

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Various non-mammalian model systems are being explored in the search for mechanisms of learning and memory storage of sufficient generality to contribute to the understanding of mammalian learning mechanisms. The terrestrial mollusk *Limax maximus* is one such model system in which mammalian-quality learning has been documented using odors as conditioned stimuli. The *Limax* odor information-processing circuits incorporate several system design features also found in mammalian odor-processing circuits, such as the use of cellular and network oscillations for making odor computations and the use of nitric oxide to control network oscillations. Learning and memory formation has been localized to a particular central circuit, the procerebral lobe, in which selective gene activation occurs through odor learning. Since the isolated *Limax* brain can perform odor learning in vitro, the circuits and synapses causally linked to learning and memory formation are assessable for further detailed analysis.

Although mollusks and mammals diverged from their common ancestor some 600 million years ago (Runneger and Pojeta 1985), there is ample evidence for a commonality of solutions used to solve analogous problems between the two taxa. This is evident in several aspects of learning and memory mechanisms, including conserved genes (Nakaya et al. 2001; Fukunaga et al. 2006), cellular signaling processes (Bailey et al. 1996; Hatakeyama et al. 2000, 2004a,b; Bukanova et al. 2005; Grinkevich et al. 2008), mechanisms of neural integration (Gelperin 1999, 2006; Antonov et al. 2003; Apfelbach et al. 2005), synaptic plasticity (Frank and Greenberg 1994; Kandel 2001; Pittenger and Kandel 2003; Gланzman 2006), and forms of learning (Delaney and Gelperin 1986; Sahley 1990; Sahley et al. 1990; McComb et al. 2005; Lorenzetti et al. 2006). These observations suggest that prospecting for mammalian memory mechanisms in the molluscan nervous system is a productive strategy, as amply demonstrated by dissection of short- and long-term memory storage mechanisms in *Aplysia* (Kandel 2001; Hawkins et al. 2006; Reissner et al. 2006).

Commonalities between mollusks and mammals have emerged in a particularly clear way from analyses of the molecular, cellular, and network dynamics associated with associative learning in *Limax maximus*, specifically in the realm of olfactory information processing and odor learning (Gelperin 2006). Vertebrate and invertebrate olfactory processing systems share several basic design features (Hildebrand and Shepherd 1997; Eis-then 2002; Ache and Young 2005), as do mammals and mollusks (Chase and Tolloczko 1993; Lledo et al. 2005). Within the gastropod mollusks, exemplified by the land snails and slugs, a common plan of olfactory system organization is evident (cf. Fig. 3.3 in Chase 2002), as is a highly developed capacity for olfactory learning (Gelperin 1975; Croll and Chase 1977, 1980; Teyke 1995; Friedrich and Teyke 1998; Ungless 1998). The following will outline some of the universal design principles displayed by the olfactory information processing system of *Limax*.

Behavioral studies of olfactory learning in *Limax*

Olfactory learning has been extensively studied in *Limax*. Aversive olfactory learning occurs when an odorant, which is the conditioned stimulus, is paired with an aversive unconditioned stimulus (Gelperin 1975; Sahley et al. 1981). For the conditioned stimulus (CS), natural food odor, such as carrot and cucumber, or a pure chemical is used. For the unconditioned stimulus (US), quinidine, carbon dioxide, or electrical shock is used. Long-lasting memory is formed after single paired presentation of CS and US, and it is retained for up to several weeks. Performance of learning is measured either as the rate of the slug's choice of the CS, or the time to approach the CS.

Short-term and long-term memories are discriminated by their dependence on protein synthesis (Matsuo et al. 2002). Short-term memory formation does not require protein synthesis, whereas long-term memory formation requires protein synthesis. After injection of anisomycin, an inhibitor of protein synthesis, into the body cavity (0.2 mg/g body weight), the peak inhibition rate of protein synthesis was ~80% in the brain. In the slugs injected with anisomycin before conditioning, memory at 24 h after conditioning was still intact, but at 48 h after conditioning, memory was lost. Another protein synthesis inhibitor, cycloheximide (1 mg/g body weight), had a weaker effect on protein synthesis (~50% inhibition), and the slugs injected with cycloheximide showed intact memory at 2 d, but memory was lost at 1 wk. These results suggest that the lifetime of short-term memory is ~1–2 d, and this may depend on the extent to which protein synthesis is inhibited (Yasui et al. 2004).

Serotonin has been proven to be necessary for aversive olfactory learning (Shirahata et al. 2006). A serotonergic neurotoxin, 5,7-dihydroxytryptamine (DHT), is incorporated into serotonergic neurons by serotonin transporters and causes a gradual decrease in the serotonin content of the neurons. The serotonin content in the central ganglia was ~40% of the normal level in 5 d after the injection of DHT (1 mg/g body weight). It may reduce the serotonin content at synaptic terminals to an even lower level and strongly block serotonergic transmission. In the slugs injected with DHT 5 d prior to conditioning, acquisition of short-term memory (tested on the next day) was blocked.

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However, it had no effect on long-term memory, which was tested 5 d after conditioning. This suggests that short-term and long-term memories have different dependence on serotonin and involve independent neural pathways. Injection of DHT after conditioning and testing 5 d later had no effects, indicating that DHT does not block retrieval of memory.

Aversive olfactory learning has been used to examine the slug's ability to discriminate odors (Teyke and Gelperin 1999; Sakura et al. 2004). First, the slug was conditioned with octanol, and on the next day, behavior to the conditioned odor and other odors was tested. As expected, most slugs showed avoidance to the conditioned odor, octanol, and did not avoid methylcyclohexanol, which is a structurally dissimilar molecule to octanol. However, a small number of slugs also avoided hexanol, a structurally similar molecule to octanol. Injection of N-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase, before testing enhanced the rate of avoidance to hexanol. L-NAME did not affect avoidance behavior to octanol. These results suggest that nitric oxide is involved in accurate discrimination of similar odors.

Higher-order conditioning

In designing experiments to probe the complexity of *Limax* odor learning, it has proven remarkably productive to regard *Limax* as possessed of mammalian learning skills in the domain of associative learning about odorants as conditioned stimuli and aversive tastes as unconditioned stimuli (Gelperin 1999). In particular, second-order conditioning and the Kamin blocking effect (Kamin 1969) were early striking examples of higher-order conditioning shown robustly in *Limax* odorant conditioning (Sahley 1990).

Second-order conditioning is obtained when an initially attractive odor (CS1) is paired with an aversive taste in phase one of conditioning, and then in phase two of conditioning CS1 is paired with a second initially attractive odor (CS2). The result is that both CS1 and CS2 become aversive (Sahley et al. 1981). Use of cooling-induced amnesia during second-order conditioning in *Limax* showed that sequential presentation of CS1 and CS2 and simultaneous presentation of CS1 and CS2 make different memories (Sekiguchi et al. 1994).

We have recently revisited the question as to how the blocking phenomenon, which requires that conditioned stimuli acquire predictive relationships with unconditioned stimuli (Fanselow and Poulos 2005), can be implemented logically and with plausible neurophysiological mechanisms (Gelperin et al. 1986; Goel and Gelperin 2006), a subject of continuing interest in the mammalian (Jennings and Kirkpatrick 2006; Padlunbaya et al. 2006) and insect (Guerrieri et al. 2005; Blaser et al. 2006) CNS.

The blocking phenomenon involves two phases of conditioning. In the first phase of conditioning, a conditioned stimulus (CS1) is paired with the unconditioned stimulus (US). In the second phase of conditioning, a compound stimulus of CS1 and a second conditioned stimulus (CS2) is presented followed by the US. Blocking is evidenced by lack of association between CS2 and the US (Sahley et al. 1981), as found in other invertebrates (Guerrieri et al. 2005) and mammals (Giannaris et al. 2002); but see Farley et al. (2004).

Our circuit solution to the blocking problem in *Limax* requires a facilitator neuron (FN) interposed between sensory inputs from CS1, CS2, and US, on the one hand, and the motor-neurons executing the conditioned response. It also requires reciprocal inhibition between the CS1, CS2, and US inputs, which can be amplified by learning via activation of the FN. These interactions were explored in simulations based on an in-

tegrate-and-fire model used previously for spiking cortical neurons and synaptic connections that can show potentiation (Goel and Gelperin 2006). There are several cellular loci in *Limax* that can potentially provide insight into the predictions of the proposed mechanism for blocking, including the serotonergic metacerebral giant cell (Kandel and Tauc 1966a,b; Gelperin 1981; Shirahata et al. 2004; Jacklet et al. 2006), the feeding command neurons (Delaney and Gelperin 1990a,b,c), and the recently identified odor-responsive visceral neuron *p*-VN (Inoue et al. 2004). The recent demonstration of odor conditioning by the isolated *Limax* CNS (Inoue et al. 2006) greatly augments the feasibility of these experiments.

Serotonergic modulation of sensory neurons is a central element in plasticity of the *Aplysia* gill- and siphon-withdrawal circuit (Glanzman 2006; Hawkins et al. 2006; Song et al. 2006), while depletion of serotonin impairs short-term but not long-term memory in *Limax* (Shirahata et al. 2006). While slugs will quickly learn to avoid a diet devoid of the essential amino acid tryptophan (Delaney and Gelperin 1986; Cook et al. 2000), if a tryptophan-deficient diet is the only food available, slugs will maintain normal levels of brain serotonin and function of the serotonergic metacerebral cell (Gietzen et al. 1992). In mammals, cells in the anterior piriform cortex are sensitive to deficiencies in essential amino acids, based on uncharged transfer RNA inducing phosphorylation of eukaryotic initiation factor 2 via a general control non-de-repressing 2 (GCN2) kinase (Gietzen et al. 2004; Hao et al. 2005; Sharp et al. 2006). The protein kinase GCN2 is an essential component of the general amino acid control (GAAC) response, an evolutionarily ancient anabolic pathway that is activated when eukaryotic cells are deprived of amino acids (Malmberg and Adams 2008). The GAAC response results in cellular anabolic processes that are exquisitely sensitive to amino acid availability. The findings of Hao et al. (2005) in mammalian piriform cortex suggest that the search for central neurons in *Limax* responding to intake of diets deficient in amino acids essential for *Limax* growth should focus on neurons showing high levels of GCN2 activation after intake of the methionine-deficient diet.

Flexible storage of compound odor stimuli

The issue of how compound odor stimuli are stored in the CNS can be explored by training animals to associate an aversive US with a compound CS under conditions in which they experience the compound of CS1 and CS2 as a spatially and temporally uniform mixture or as a spatially and temporally heterogeneous mixture (Hopfield and Gelperin 1989). The heterogeneous mixture of CS1 and CS2 provides information on the components of the mixture as well as the mixture itself. The uniform mixture is more likely to be treated as a single odor object. A difference in storage mechanism is revealed by measuring the aversion to CS1 and CS2 presented alone after aversive compound conditioning to the mixture. *Limax* appears to store a uniform mixture of CS1 and CS2 as a unique odor object such that strong aversive responses are shown to the CS1 + CS2 compound, but clear attraction to CS1 alone and CS2 alone remains. Conversely, pairing the US with a heterogeneous mixture of CS1 and CS2 leads to a different form of sensory storage because after conditioning, slugs show strong aversion to CS1 presented alone and CS2 presented alone, as well as strong aversion to the compound stimulus (Hopfield and Gelperin 1989). Similar results were obtained using cooling-induced retrograde amnesia to probe the nature of CS storage after compound conditioning. Using cooling to alter stimulus representations, slugs appeared to learn a binary odor mixture as a unitary object when they had no exposure to the separate elements of the mixture (Sekiguchi et al. 1999). These

experiments emphasize the synthetic role of olfactory processing and the critical role of learning in stimulus processing (Wilson and Stevenson 2003).

Neural systems for olfactory processing

Limax has two pairs of tentacles, the superior and inferior tentacles that sense odors. Each tentacle has a sensory epithelium at its tip and a tentacular ganglion (TG) beneath it. The TG is the primary center of olfactory information processing. The TG contains interneurons, as well as projection neurons that project to the cerebral ganglion (Ito et al. 2000). The TG shows a spontaneous oscillation of local field potential (LFP) at ~2 Hz (Ito et al. 2003a). The oscillation is modulated by odors and wind (Ito et al. 2006a). Several neurotransmitters, such as serotonin, acetylcholine, glutamate, and GABA, exist in the TG and modulate the spontaneous oscillation (Inokuma et al. 2002; Ito et al. 2003b, 2004). In addition, the sensory epithelium also shows an oscillatory electro-olfactogram (EOG) in response to olfactory stimulus. The EOG oscillation interacts with the LFP oscillation in the TG (Ito et al. 2006a).

TG neurons project to the cerebral ganglion via the tentacle nerves. The major target of projection is the procerebral (PC) lobe, which is the lateral part of the cerebral ganglion. Recent results show that either the superior or inferior tentacles alone can be used for the acquisition and retrieval of aversive olfactory memory (Kimura et al. 1999; Yamagishi et al. 2008).

The procerebrum, or PC, lobe is a division of the cerebral ganglion unique to terrestrial slugs and snails that is specialized for the processing of olfactory information (Ratté and Chase 1997, 2000; Chase 2000; Zaitseva 2000). The PC lobe consists of three layers, the cell mass (CM), terminal mass (TM), and internal mass (IM). The CM contains a large number of cell bodies of neurons, which are generally small (5–8 μ m) (Ratté and Chase 1997). The TM and IM are neurophil layers. The TM receives input from the tentacle nerve (Kawahara et al. 1997). The PC lobe spontaneously produces a periodic slow oscillation of LFP (~0.7 Hz) (Gelperin and Tank 1990). The LFP oscillation is well synchronized over the entire PC lobe, but the phase of the oscillation is advanced at the apical end. This results in periodic propagating waves of neural activity from the apical to basal ends, which is clearly shown by optical imaging (Delaney et al. 1994; Kleinfeld et al. 1994; Kawahara et al. 1997; Inoue et al. 1998).

Patch clamp recording from single PC neurons showed that the neurons in the PC lobe are categorized as either bursting (B) or nonbursting (NB) neurons (Delaney et al. 1994; Kleinfeld et al. 1994). B neurons are characterized by periodic bursting activity, and NB neurons are silent or fire at a low frequency. The spontaneous bursting activity of B neurons and inhibitory synaptic potentials in NB neurons are synchronized with the LFP oscillation.

B neurons are a few percent of the total neuronal population and are coupled with each other by chemical and electrical synapses (Ermentrout et al. 2004). B neurons have extensive synaptic connections with NB neurons (Wang et al. 2001). Stimulation of the superior tentacle with odorants results in inhibitory responses in 45% of NB neurons, while 11% of NB neurons show an excitatory response (Murakami et al. 2004). The periodic membrane potential trajectories of B and NB cells lead to a phase-dependent response to sensory inputs (Inoue et al. 2000). Both B and NB cells contain the mRNA for nitric oxide synthase and soluble guanylate cyclase (Fujie et al. 2005), critical to the control of oscillation frequency in the PC lobe (see below). The B cells located in the apical region of the PC lobe are more excitable (shorter interburst interval) than the B cells located in the basal region of the PC lobe (Ermentrout et al. 1998). Chloride-

dependent periodic depolarizations in apical B cells have higher amplitude than that recorded from B cells in the basal region of the PC lobe (Watanabe et al. 2003). This gradient of excitability and pattern of connectivity give rise to activity waves in the PC lobe traveling from apex to base at 1.1 mm/sec at a repetition rate of 0.7 Hz in vitro (Delaney et al. 1994; Kleinfeld et al. 1994). These traveling waves have also been found in mammalian cortex, although their computational role is still unclear (Ermentrout and Kleinfeld 2001). Evidence for traveling waves in the PC lobe of intact behaving slugs is suggestive but not definitive (Cooke and Gelperin 2001).

Calcium imaging of NB neurons filled with the calcium sensitive dye rhod-2 showed calcium transients in response to electrical stimulation of the tentacle nerve or, in some cases, stimulation of the tentacle with an odorant. If multiple stained NB cells responded, they were synchronized with each other (Watanabe and Kirino 2007). The importance of activity-dependent calcium transients in B and NB cells is also shown by the effects of caffeine application, which enhances cytoplasmic calcium concentrations in isolated PC cells in a ryanodine-sensitive mechanism. Caffeine also elevates the frequency and amplitude of the oscillation in local field potential in the PC lobe, but with a time lag between these two effects, suggesting two distinct mechanisms for the two effects (Liu et al. 2007).

Electrophysiological analysis revealed that the TN makes direct connection with NB neurons, but not directly with B neurons (Inoue et al. 2000). Intracellular dye injection revealed that B neurons are multipolar and have projections within the CM, while the NB neurons are monopolar and have projections in the neuropil layers of the PC lobe (Watanabe et al. 1998). The morphology of the neurons is consistent with the electrophysiological data, because both NB neurons and the fibers from the tentacular nerve project to the TM, but B neurons do not. The neurites of B neurons project along the dorsal-ventral axis of the PC lobe. This is the direction perpendicular to the propagation of waves, and parallel to the orientation of the cell clusters activated in synchrony. The projection pattern of the neurites of B neurons may therefore serve to make a population of PC neurons be activated synchronously (Fig. 1).

There are direct interactions between the oscillatory circuitry in the PC lobe and motoneurons controlling nose posi-

Figure 1. A diagram of the intrinsic connectivity of bursting and non-bursting neurons in the *Limax* procerebral lobe and their relations to olfactory input pathways and output pathways to locomotor and other integrative centers.

tioning (Nikitin et al. 2005) and remote neurons in the pedal and buccal ganglia (Gelperin and Flores 1997). A behavioral interaction between olfactory inputs and feeding motor responses has been documented (Sahley et al. 1992). Other regions of the cerebral ganglion adjacent to the PC lobe may also show oscillatory local field potentials (Watanabe et al. 2004). Odor-elicited changes in oscillatory activity in the PC lobe of freely behaving *Helix* have recently been described (Samarova and Balaban 2007).

The structure of the network of the PC lobe seems essentially similar to that of the mammalian brain, such as olfactory bulb or cortex, which is usually composed of projection neurons and local interneurons. It has been speculated that NB neurons, which presumably correspond to projection neurons, may have principal roles in encoding olfactory information, while B neurons may regulate the activity level of NB neurons. In response to olfactory stimulus to the tentacle, NB neurons showed either excitatory or inhibitory responses, and the type of the response was largely specific to the odor (Murakami et al. 2004). In theory, this allows the population of NB neurons to encode odor identity. On the other hand, the LFP frequency, which presumably reflects the activity level of B neurons, regulates the activity level of NB neurons, apparently suppressing NB neurons with its higher frequency (Murakami et al. 2004).

Evidence indicates that B neurons release glutamate that inhibits NB neurons (Kleinfeld et al. 1994), although synaptic connections between B and NB neurons have not been proven by direct electrophysiological recording. Voltage clamp analysis using perforated patch recording showed that puff-applied glutamate opens potassium (K) channels in NB neurons, and quisqualate mimics this effect (Watanabe et al. 1999). Glutamate also opens chloride (Cl) channels in B neurons, and ibotenate mimics this effect (Watanabe et al. 1999). The glutamate-activated Cl channel in B neurons has excitatory actions, because ibotenate activates the LFP oscillation and increases the cytoplasmic calcium concentration in a fraction of cultured PC neurons. This channel seems to be more densely expressed in the B neurons near the apical end of the PC lobe than those near the basal end, and this may underlie the wave propagation from the apical to basal ends (Watanabe et al. 2003).

Electrical synapses may exist between B neurons, since octanol and glycyrrhetic acid, which block electrical synapses, block the LFP oscillation (Ermentrout et al. 2004). Acetylcholine may be another transmitter mediating fast synaptic transmission in the PC lobe (Watanabe et al. 2001), although it is not yet clear which synapses are cholinergic. In addition to transmitters involved in fast synaptic transmissions, many kinds of neuromodulators, including monoamines, peptides, and gaseous transmitters, are found in the PC lobe, and some of them actually modulate the LFP oscillation (Gelperin et al. 1993, 2000; Gelperin 1999). The mechanisms of the LFP modulation by the neuromodulators can be diverse. For example, both NO and serotonin increase the frequency of the LFP oscillation. However, NO depolarizes the basal level of the membrane potential of B neurons, while serotonin increases the extent of depolarization and keeps the basal level unchanged (Inoue et al. 2001). Therefore, there will be multiple targets of neuromodulators in the PC lobe.

After being processed in the PC lobe, information is transmitted to other brain regions and finally elicits motor output. Because the neurons in the PC lobe exhibit clear oscillatory activity, the output neurons will receive oscillatory input. Optical recording shows coherent oscillatory activity in the cerebral ganglion outside the PC lobe (Watanabe et al. 2004). An identified neuron in the cerebral ganglion shows activities coherent with the PC lobe (Shimozono et al. 2001). Neurons in the pedal and

buccal ganglia also show coherent activity with that of the PC lobe (Gelperin and Flores 1997).

Oscillations and active sampling in olfaction

Mollusks and mammals also share the use of oscillatory dynamics in early olfactory centers (procerebral, PC, lobe in terrestrial mollusks and olfactory bulb, OB, in mammals) (Gelperin 2006). The rosy wolfsnail *Euglandina rosea* uses chemosensation for hunting its prey (Clifford et al. 2003; Shaheen et al. 2005) and has a PC lobe with oscillating local field potentials (LFPs) (Harrington et al. 2004), as in *Limax* (Gelperin and Tank 1990) and *Helix* (Nikitin and Balaban 2000; Nikitin et al. 2005). Hunting dogs use olfaction for trail following (Hepper and Wells 2005) and use active odor sampling, or sniffing (Gazit et al. 2003; Schoenfeld and Cleland 2005; Settles 2005), as an essential component of their odor sampling strategy. Analogously, snails show a variety of "rapid" tentacle (nose) movements associated with odor localization that may serve some of the same functions as sniffing (Lemaire and Chase 1998; Ungless 2001; Nikitin et al. 2005; Nikitin et al. 2008). Sniffing may be associated with taking olfactory snapshots of odorant inputs (Uchida and Mainen 2003; Uchida et al. 2006) when rapid sampling and decision-making are required (Verhagen et al. 2007; Wesson et al. 2008).

Providing motivation for learning odor associations

We have found that water deprivation is a potent motivational method for obtaining conditioned odor aversions in *Limax*. Slugs are weighed and then dehydrated by placing them in mesh cages in a container with dessicant and a fan (Fig. 2). Dehydration continues until the slugs lose 35%–40% of their initial fully hydrated body weight. Rapid dehydration produces maximally motivated slugs. Dehydrated slugs actively search for a source of water for contact rehydration (Prior 1985; Prior et al. 1994; Matanock and Welsford 1995), which occurs when the slug sits on a moist surface and absorbs water through its foot via a paracellular pathway (Prior 1984; Prior and Uglem 1984; Banta et al. 1990). During training, dehydrated slugs are first offered salt water containing quinine and NaCl for rehydration, paired with odor A. Contact of the slug with the salt water elicits rapid withdrawal. Slugs are then offered safe pure water paired with odor B. Odor sources are placed under the mesh floor of the training chamber with the water source located directly above the odor source. The slug is given 30 min to rehydrate on the moist surface above odor B. The next day, slugs are dehydrated again and positioned between sources of odor A and odor B located under the mesh floor of the testing chamber. Latency to approach an odor source and time spent within a 9-cm circle centered on each odor source are recorded. In three experiments with six different odorants in which 31 slugs were trained with odor A signaling safe water and odor B signaling salt water, 90% of trained slugs chose odor A. Since slugs can be dehydrated in 40–60 min and survive repeated bouts of dehydration after contact rehydration, the method of water–odor pairing during contact rehydration is much more convenient than food deprivation, which can require many days to re-establish appetitive odor responses after feeding to satiation.

We and others (Prior 1984, 1985; Banta et al. 1990; Matanock and Welsford 1995) have observed *Limax* orient and move on a dry surface toward a source of water, prior to initiating contact rehydration. It is a mystery how an animal with a completely moist surface can show directed orientation to a localized source of water containing no explicit added odor cue, as dehydrated slugs are able to do.

Figure 2. Method for appetitive conditioning of odor preferences using dehydration followed by water reward. (A) The slug is dehydrated to 60%–65% of its fully hydrated weight within 30 min. (B) The slug is offered dangerous water with odor A. (C) The slug is offered safe water with odor B. (D) The slug is again dehydrated. (E) The dehydrated slug is offered a choice between spending time over odor A or odor B.

Neurogenesis in olfactory pathways

Another design principle of olfactory information processing shared between mammals and mollusks is the addition of new circuit elements post-hatching or post-parturition to both the sensory periphery (Graziadei and Graziadei-Monti 1979; Chase and Rieling 1986) and the central processing structures—PC lobe (Zakharov et al. 1998) and OB (Magavi et al. 2005; Lledo and Lagier 2006; Lledo et al. 2006). To investigate the locus and extent of neurogenesis in the *Limax* PC lobe, newly hatched slugs (30–40 mg) were immersed in a saline solution containing bromodeoxyuridine (BrdU; 5 mg/mL) for 90 min. After BrdU treatment, slugs were rinsed with *Limax* saline and placed in containers with a moist substrate and their maintenance diet, rat chow. At various periods after BrdU treatment, brains were harvested and processed as whole mounts for immunocytochemical demonstration of BrdU incorporation into DNA of dividing cells, which are presumed to be mostly neurons. The CNS was processed with a protocol supplied with the BrdU in situ detection kit. Briefly, tissue was fixed, treated with detergent, taken to pH 6 at 89°C to open DNA strands, and treated with biotinylated anti-BrdU antibody and streptavidin-HRP secondary antibody. Preparations were developed with diaminobenzidine as the chromogen and mounted in glycerol prior to digital photography. Figure 3A shows the PC lobe from a slug given a BrdU bath 3 d post-hatching and the PC lobe harvested 1 d later and developed to reveal BrdU-labeled neurons. The zone of neurogenesis is clearly visible at the apical pole of the PC lobe, which is also the

site of initiation of activity waves that traverse the apical–basal axis at 0.7 Hz in isolated preparations (Delaney et al. 1994; Kleinfeld et al. 1994; Toda et al. 2000; see also Nikitin and Balaban 2000). Figure 3B shows the band of neurogenesis revealed in the PC lobe treated to reveal neuronal BrdU incorporation 2 mo after treatment of the hatchling slug. As the apical neurons in the zone of neurogenesis shown in Figure 3A continue to divide, the more apical of the daughter neurons continue to divide so that the original BrdU-labeled neurons are left behind and the apical region of the PC lobe extends, a hypothesis that accounts well with previously published patterns of neurogenesis in the PC lobe (Zakharov et al. 1998). Since the period of active neurogenesis occurs post-hatching when the animal would be exposed to natural olfactory stimulation, it is possible that the diversity and intensity of olfactory experience, and perhaps odor learning, modulate the rate and extent of neurogenesis in the PC lobe and perhaps neuronal survival. Enhanced survival of new neurons in the mammalian olfactory bulb due to enriched odor stimulation has been documented, with a concomitant enhancement of short-term odor memory (Rochefort et al. 2002; Rochefort and Lledo 2005; Woo et al. 2006).

Procerebrum as an olfactory learning center

Increasing evidence indicates that the PC lobe is involved in odor learning in *Limax*. Optical studies of odor-elicited activity in the

Figure 3. Zones of neurogenesis in the procerebral lobe are revealed by bromo-deoxyuridine (BrdU) labeling. (A) PC lobe of a slug exposed to BrdU at 3 d post-hatching and BrdU staining developed 1 d later. (B) PC lobe of a slug exposed to BrdU at 3 d post-hatching and BrdU staining developed 2 mo later. The apical region of the PC lobe is a site of continued neurogenesis over this period.

PC lobe showed changes due to prior conditioning of responses to an odorant (Nikitin and Balaban 2000). Wavelet-based analysis may help to characterize more completely the complex dynamics of local field potential activity as modified by odor input in the PC lobe (Schutt et al. 2002, 2003). A small set of genes is specifically activated in PC lobe neurons owing to odor learning (Nakaya et al. 2001). Antibodies to the gene product for one of the learning-activated genes have been made and used to show the enhanced levels of expression of the gene after odor learning.

A direct test of the role of the PC lobe in odor learning in *Limax* is based on studies of the effects of lesions of the PC lobe on subsequent odor learning. Ablation of the PC lobe 7 d prior to odor conditioning produced clear deficits in odor conditioning not evident in slugs given control operations (Kasai et al. 2006). PC lobe ablation after odor conditioning also decremented avoidance of the aversively conditioned odor. Control experiments showed that PC lesioned slugs were not anosmic (Kasai et al. 2006). These results strongly support evidence from optical (Kimura et al. 1998b; Toda et al. 2000), molecular, and activity-dependent dye uptake studies (Kimura et al. 1998c; Sekiguchi et al. 2008), also indicating a selective role of the PC lobe in odor learning.

Other genes have been identified that are activated by noxious stimuli like those used as the US in odor conditioning. One kruppel-like factor (KLF) family transcription factor gene (*Limax* KLF; *limKLF*) is up-regulated 30 min after depolarization of the isolated CNS with high potassium saline (Fukunaga et al. 2006). An immediate early gene, CCAAT enhancer-binding protein (C/EBP) was cloned by reverse transcription-polymerase chain reaction (RT-PCR). C/EBP was also activated by noxious electrical stimuli applied to the *Limax* body wall or by the aversive taste of quinidine (Fukunaga et al. 2006), used as the US in many odor conditioning experiments with *Limax* (Yamada et al. 1992; Sekiguchi et al. 1994; Matsuo et al. 2002; Yasui et al. 2004).

The tentacular ganglion located just behind the nose in the superior tentacles also shows oscillatory local field potentials (Inokuma et al. 2002). Odor-elicited uptake of a fluorescent derivative of glucose has been demonstrated in the tentacular ganglion (Ito et al. 2006b). The oscillatory activity of the tentacular ganglion is modulated by acetylcholine, serotonin, and γ -aminobutyric acid (Ito et al. 2004) as identified immunocytochemically (Ito et al. 2003a). Odor-elicited spike activity can be recorded in the tentacular nerve and correlated with oscillations in LFP (Ito et al. 2003b). Recent work shows that the summed receptor potentials of the olfactory receptors, recorded as the electro-olfactogram (EOG), can also show oscillations (Ito et al. 2006a), as previously found in several vertebrate olfactory epithelia (Dorries and Kauer 2000; Nikonov et al. 2002; Suzuki et al. 2002, 2004). How the interactions of the peripheral EOG oscillation and the central LFP oscillation enhance odorant detection remains to be determined.

A variety of other cells and neurotransmitters have been shown to play some role in odor aversion learning by *Limax*. The *p*-VN neuron was first identified as an output neuron whose activity could be used to provide an index of aversive evaluation of odor input (Inoue et al. 2004). Monitoring activity in *p*-VN was crucial to the demonstration of in vitro odor learning by the isolated *Limax* CNS (Inoue et al. 2006). Among the 21 neurotransmitters known to play a role in *Limax* odor processing (Gelperin 1999), serotonin has been shown to play an essential role in odor learning (Shirahata et al. 2004, 2006). The peptide FMRFamide may play a regulatory role in stimulating NO production by the neuronal nitric oxide synthase (NOS) containing neurons in the PC lobe (Roszer et al. 2004; Watanabe and Kirino 2004). Nitric oxide plays a crucial role in appetitive conditioning but not in aversive conditioning (Yabumoto et al. 2008). How-

ever, odor discrimination can be blocked by pharmacological block of NOS (Sakura et al. 2004) in the aversive conditioning paradigm. Nitric oxide plays a major role in controlling network dynamics in the *Limax* olfactory system both centrally and peripherally (Fujie et al. 2002, 2005). NO is also a modulator of chemosensory processing in *Aplysia* (Moroz 2006). Serotonin and NO have complementary effects on elements of the olfactory processing network in *Limax* (Inoue et al. 2001).

Mechanisms of learning and memory

Bilateral ablation of the PC lobe, either before or after conditioning, resulted in impairment of memory (Kasai et al. 2006). However, ablation of the PC lobe did not affect detection of odors, because the ablated slugs showed normal avoidance to the innately aversive odor of garlic. Lucifer yellow injected immediately after conditioning stained a cluster of neurons in the PC lobe (Kimura et al. 1998c; Ermentrout et al. 2001; Sekiguchi et al. 2008). The staining was selectively observed in the PC of the conditioned animals and has been suggested to reflect elevated neural activity. The cluster was observed in just one of the two PC lobes and often formed a band along the dorsal-ventral axis. Optical recording using a voltage-sensitive dye revealed an elevated activity in the PC lobe in response to conditioned odor (Kimura et al. 1998b).

Learning-related neural activity has been observed by conditioning in isolated tentacle-brain preparations. First, a neural correlate of aversive behavior in response to olfactory stimuli has been identified (Inoue et al. 2004). The posterior visceral neuron (*p*-VN) is a motor neuron innervating the mantle muscle and mediates mantle contraction response in response to aversive odor stimuli. The *p*-VN is activated by aversive odors. It is activated not only by innately aversive odors, but also by aversively conditioned odors. The activity of this neuron can therefore be used to monitor putative conditioned response, after in vitro conditioning in isolated tentacle-brain preparations. In vitro conditioning was done by applying an odor to the tentacle, followed by electric stimulation of the lip nerve, which is a substitution of aversive taste stimulus. After the paired stimuli, the *p*-VN fired more strongly to the conditioned odor, while the response to unpaired odors remained unchanged (Inoue et al. 2006).

After conditioning in the nose-brain preparation, the frequency of the LFP oscillation in the PC lobe increased more strongly in response to aversively conditioned odors, while weaker responses were observed with unpaired odors (Inoue et al. 2006). These results indicate a link between the frequency of the LFP oscillation and the aversiveness of odors. Similar frequency changes after conditioning have been reported in a preparation made after conditioning in vivo (Kimura et al. 1998a).

Molecular biology provides very useful tools to study mechanisms of learning. By the mRNA differential display technique, a gene was found whose expression in the PC increases after olfactory aversive learning (Nakaya et al. 2001). This gene, named learning-associated protein of the slug at 18 kDa (LAPS18), encodes a peptide of 18 kDa, which is secreted to the extracellular space and promotes cell aggregation and neurite extension. Homologs of this gene are found in both vertebrates and invertebrates. One such homolog in *Aplysia* named *Aplysia* LAPS18-like protein (ApLLP) plays critical roles in the reflex learning of gill withdrawal (Kim et al. 2006). Studies of transcriptional activators required for establishing long-term memory (Liu and Song 2008) and the role of NO in memory storage (Susswein et al. 2004; Yabumoto et al. 2008) continue to highlight mechanistic commonalities between molluscan and mammalian learning mechanisms (Yasui et al. 2004).

Conclusion

Results from the *Limax* system were some of the first to draw attention to the potential computational role of coherent oscillatory activity in neural networks engaged in olfactory information processing (Gelperin and Tank 1990; Tank et al. 1994; Gelperin 1999). Coherent network oscillations in central olfactory networks are now recognized as an essential feature of the computations performed by these circuits (Laurent and Davidowitz 1994; Laurent and Naraghi 1994; Gelperin 2006). Initial observations of coherent oscillations in the *Limax* central olfactory circuit have since been extended to determine the integrative role of bursting and nonbursting olfactory interneurons during each cycle of the network oscillation that result in phase-dependent filtering of sensory inputs (Inoue et al. 2000). The role of the gaseous neurotransmitter nitric oxide in modulating central olfactory processing and odor learning was also an early result in the *Limax* system (Gelperin 1994) that presaged a great deal of similar work in other olfactory systems, both invertebrate (Nighorn et al. 1998; Katzoff et al. 2002; Muller and Hildebrandt 2002; Wilson et al. 2007) and mammalian (Kendrick et al. 1997; Lowe et al. 2008). We now know that the effects of nitric oxide in the *Limax* olfactory system are exerted on appetitive but not aversive olfactory learning (Yabumoto et al. 2008). The *Limax* system also provided early indications that invertebrate olfactory systems display the same features of higher-order conditioning previously known from studies of mammalian conditioning (Gelperin 1975; Sahley et al. 1981; Hopfield and Gelperin 1989; Sahley 1990; Sekiguchi et al. 1994, 1997, 1999). Finally, the *Limax* system also provided a particularly clear example of the creative interplay between computational modeling and experimental measurements in olfactory systems (Gelperin et al. 1986, 1989; Ermentrout et al. 1998, 2001, 2004; Goel and Gelperin 2006), which is now recognized as an essential aspect of the functional dissection of olfactory information processing systems (Laurent 2002; Brody and Hopfield 2003; Cleland and Linster 2005; Koulakov et al. 2007).

As outlined above, the circuits and synapses causally related to odor learning are accessible to functional analysis given the demonstration of odor learning by the isolated nose-brain preparation of *Limax* in vitro (Inoue et al. 2006). An important component of the odor learning is localized to the procerebral lobe (Kasai et al. 2006), whose constituent neurons are amenable to both electrophysiological (Delaney et al. 1994; Kleinfeld et al. 1994; Watanabe et al. 1999; Murakami et al. 2004) and optical (Wang et al. 2003; Watanabe et al. 2004; Watanabe and Kirino 2007) recording. The stage is set for isolating and manipulating the critical cellular events underlying odor learning to move the analysis of events in the *Limax* odor learning system from the study of correlates of learning to the study of causative events for learning.

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